

**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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The University of Dublin

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By

Teresa Ann Burke

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Supervised by

Prof Andrew Harkin
and
Dr Neil Upton

School of Pharmacy and Pharmaceutical Sciences
&
Trinity College Institute of Neuroscience,
Trinity College, Dublin
Volume I

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, except for the following contributions:

Chapter 3

Dr Danka Kozareva assisted in the performance of the surgeries.

Chapter 4

Dr Danka Kozareva performed the surgeries in 6.5 and 13 week studies and assisted in the performance of the behavioural testing throughout. Dr Danka Kozareva, Mr Matthew McAuslan and Ms Caoimhe Tyndall assisted in the processing of the brain tissue throughout.

Dr Danka Kozareva and Mr Matthew McAuslan performed the immunofluorescence and immunofluorescence imaging throughout. Ms Almudena Otalora Alcaraz performed the immunohistochemistry for the 6.5 study under the supervision of Dr Danka Kozareva. Ms Caoimhe Tyndall assisted with the immunohistochemistry for the 13 week study.

Chapter 5

Dr Danka Kozareva performed half of the surgeries. Dr Danka Kozareva performed the immunofluorescence and Dr Danka Kozareva and Mr Matthew McAuslan performed the immunofluorescence imaging. Mr Matthew McAuslan assisted in the processing of the brain tissue. Dr Danka Kozareva and Mr Matthew McAuslan assisted in the performance of behavioural testing. Ms Caoimhe Tyndall assisted with the immunohistochemistry and tissue collection.

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Signed

Teresa Burke

*This thesis is dedicated to my
Aunty Marjorie Higgins nee McCluskey*

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Table of Contents

Declaration (ii)	
Acknowledgements (iv)	
Table of Contents (vi)	
List of Figures (x)	
List of Tables (xvi)	
List of Abbreviations (xvii)	
Abstract (xx)	
Summary (xxii)	

1. Introduction.....	1
1.1 Parkinson's disease	1
1.1.1 Treatments for Parkinson's disease	3
1.1.2 Environmental Toxins and their impact on Parkinson's disease pathology	4
1.1.3 Parkinson's disease and inflammation	5
1.1.4 Sex differences in Parkinson's disease.....	5
1.1.5 Parkinson's disease genetics.....	6
1.2 Alpha-synuclein	8
1.2.1 Alpha-synuclein's physiological role.....	8
1.2.2. Alpha-synuclein conformations.....	9
1.2.3 Alpha-synuclein and Parkinson's disease.....	10
1.2.4 Alpha-synuclein conformations in disease.....	11
1.2.5 Lewy Bodies	12
1.2.6 Cell to cell spreading of alpha-synuclein in Parkinson's disease	14
1.2.7 Synucleinopathies	15
1.2.8 Alpha-synuclein interactions with cellular components in disease.....	17
1.2.9 Alpha-synuclein and synaptic vesicle trafficking in disease	20
1.2.10 Alpha-synuclein and mitochondrial function in disease	21
1.2.11 Why are dopaminergic neurons vulnerable?	22
1.2.12 Alpha-synuclein and neuroinflammation.....	23
1.2.13 Interplay of alpha-synuclein with amyloid β and tau	24
1.2.14 Alpha-synuclein and aging	24
1.2.15 Targeting alpha-synuclein therapeutically.....	25
1.3 Animal models of Parkinson's disease.....	28
1.3.1 Chemically induced models	29
1.3.2 Transgenic models.....	30
1.3.3 Use of alpha-synuclein derived from humans to generate animal models of alpha-synucleinopathy.....	32
1.3.4 AAV models of disease	34
1.3.5 Preformed fibrils models of disease.....	40
1.3.6 Caveats when interpreting results from preclinical studies utilising alpha-synuclein	41
1.4 Objectives of the thesis	43
2. Materials and Methods.....	44
2.1 Materials	44
2.2 Methods	48
2.2.1 Animals.....	48
2.2.2 Adeno-Associated Viral (AAV) Vectors	48
2.2.3 Pre-Formed Fibrils (PFFs).....	51
2.2.4 Stereotaxic Surgery	52
2.2.5 Behavioural Testing.....	55
2.2.6 Immunohistochemistry.....	64
2.2.7 Immunofluorescence.....	66
2.2.8 Image Analysis.....	68
2.2.9 Statistical Analysis	70

3. Assessing the implications of expression of wildtype human alpha-synuclein in the rat substantia nigra through the use of adeno-associated viral vectors.....	73
3.1 Introduction.....	73
3.2 Study Aims and Objectives.....	78
3.3 Methods	79
3.3.1 Justification of Study Design.....	79
3.3.2 Experimental Timeline	80
3.3.3 Experimental Design	82
3.3.4 Adaptations to protocols	83
3.4 Results – 12 Week AAV-WT Study.....	85
3.4.1 Alpha-synuclein Immunofluorescence.....	85
3.4.2 GFP Immunofluorescence	86
3.4.3 Staircase Test.....	87
3.4.4 Cylinder Test.....	89
3.4.5 Tyrosine Hydroxylase Immunoreactive Cells.....	97
3.4.6 Summary of results – 12 Week AAV-WT Study	98
3.5 Results – 16 week AAV-WT Study	99
3.5.1 Alpha-synuclein Immunofluorescence.....	99
3.5.2 Staircase Test.....	101
3.5.3 Cylinder Test.....	109
3.5.4 Amphetamine Rotation Test	116
3.5.5 Open Field Test.....	121
3.5.6 Summary of results – 16 week AAV-WT Study	122
3.6 Discussion	123
4. 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.	127
4.1 Introduction.....	127
4.2 Study Aims and Objectives.....	132
4.3 Methods	133
4.3.1 Justification of Experimental Design.....	133
4.3.2 Experimental Timeline	134
4.3.3 Experimental Design	137
4.3.4 Adaptations to protocols	138
4.4 Results – 3.5 week AAV-A53T Study	144
4.4.1 Alpha-synuclein immunofluorescence.....	144
4.4.3 Cylinder Test.....	147
4.4.4 Stepping Test.....	151
4.4.5 Staircase Test.....	156
4.4.6 Tyrosine hydroxylase immunoreactive cells.....	158
4.4.7 Summary of Results 3.5 week AAV-A53T Study	162
4.5 Results – 6.5 week AAV-A53T Study	163
4.5.1 Alpha-synuclein immunofluorescence.....	163
4.5.2 Staircase Test.....	169
4.5.3 Cylinder Test.....	173
4.5.4 Stepping Test.....	180
4.5.5. Tyrosine Hydroxylase Immunoreactive Cells.....	192
4.5.6 Summary of Results 6.5 Week AAV-A53T Study.....	194
4.6 Results – 13 week AAV-A53T Study	195

4.6.1 Alpha-synuclein immunofluorescence	195
4.6.2 Staircase Test	201
4.6.3 Cylinder Test	210
4.6.4 Stepping Test	228
4.6.5 Amphetamine Rotation Test.....	235
4.6.6 Tyrosine hydroxylase immunoreactive cells	246
4.6.7 Summary of results 13 Week AAV-A53T Study.....	248
4.7 Discussion.....	249
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	255
5.1. Introduction	255
5.2. Study Aims and Objectives	260
5.3. Methods	261
5.3.1 Justification of Experimental Design	261
5.3.2 Experimental Timeline.....	262
5.3.3 Experimental Design.....	264
5.3.4 Treatment Groups.....	265
5.3.5 Adaptations to protocols	266
5.4 Results.....	271
5.4.1 Alpha-synuclein immunofluorescence	271
5.4.2 Cylinder Test	279
5.4.3 Stepping Test	287
5.4.4 Amphetamine Rotation Test.....	299
5.4.5 Staircase Test	303
5.4.6 Tyrosine hydroxylase immunoreactive cells	307
5.4.7 Summary of Results.....	313
5.5 Discussion	315
6. Discussion.....	322
6.1 Summary of main findings.....	322
6.2 Intranasal injection of AAV5-CBA-alpha-synuclein WT does not lead to the development of motor deficits up to 16 weeks post-AAV delivery.	324
6.3 Intranasal injection of AAV1/2-CMV-A53T alpha-synuclein leads to tyrosine hydroxylase immunoreactive cells loss.	325
6.4 Intranasal injection of a combination of AAV1/2-CMV-A53T human alpha-synuclein and A53T alpha-synuclein preformed fibrils (PFFs) leads to motor deficits.	326
6.5 Male rats develop motor behavioural deficits earlier than female rats following an intranasal injection of a combination of AAV1/2-CMV-A53T alpha-synuclein and A53T alpha-synuclein preformed fibrils (PFFs).....	329
6.6 Future Directions.....	333
6.6.1 Confirmation of alpha-synuclein immunofluorescence.....	333
6.6.2 Exploration of microglial activation state following AAV + PFF combination treatment	333
6.6.3 Investigation of sex differences observed following AAV + PFF combination treatment	334
6.6.4 Assessment of pre-motor (prodromal) symptoms associated with synuclein deposition and neurodegeneration	334
6.7 Concluding Remarks	336

7.1 APPENDIX I – Additional Data:	337
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)	337
7.1 12 week AAV-WT Study	337
7.1.1 Cylinder Test – Baseline	337
7.2 16 week AAV-WT Study	339
7.2.1 Staircase Test – Baseline	339
7.2.2 Cylinder Test	341
7.2 APPENDIX II – Additional Data:	347
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)	347
3.5 week AAV-A53T Study	347
7.2.1 Baseline performance cylinder test	347
7.2.2 Baseline performance stepping test	349
7.2.3 Baseline performance staircase test	354
7.2.4 Baseline performance staircase test	356
7.2.5 Baseline performance stepping test	358
7.2.6 Baseline performance staircase test	363
7.2.7 Cylinder test 50-52 days post-AAV delivery	365
7.2.8 Cylinder test 86 days post-AAV delivery	371
7.2.9 Stepping Test Baseline	377
7.2.10 Amphetamine rotation test	383
7.3 APPENDIX III – Additional Data:	388
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)	388
7.3.1 Baseline performance cylinder test	388
7.3.2 Baseline performance stepping test	394
7.3.3 Baseline performance staircase test	400
References	402

List of Figures

Figure 1.1: The location of the substantia nigra in the human brain	3
Figure 1.2: Differences in PD symptomatology between males and females	8
Figure 1.3: Illustration of the Braak staging model	12
Figure 1.4: The main conformations of alpha-synuclein found in the brain	15
Figure 1.5: Proposed alpha-synuclein interactions with various cellular components	19
Figure 1.6: Proposed mechanisms for targeting alpha-synuclein as a potential treatment for PD	28
Figure 2.1: Shuttle plasmid maps for (A) AAV5-CBA-eGFP and (B) AAV5-CBA-alpha-synuclein (human WT)	49
Figure 2.2: Shuttle plasmid map for AAV1/2-CMV-Null/Empty	50
Figure 2.3: Shuttle plasmid map for AAV1/2-CAG-miR scrambled control alpha synuclein co-expressing eGFP	50
Figure 2.4: Shuttle plasmid map for AAV1/2-CMV-A53T alpha-synuclein human.	51
Figure 2.5: The stereotaxic coordinates for unilateral injection into the substantia nigra pars compacta for all AAV injections	53
Figure 2.6: The stereotaxic coordinates for unilateral injection into the substantia nigra pars compacta for all PFF injections	54
Figure 2.7: Staircase test of skilled motor function	56
Figure 2.8: Illustration of the performance of the stepping test	58
Figure 2.9: The cylinder test of limb use asymmetry	60
Figure 2.10: The amphetamine rotation test	63
Figure 2.11: Example of threshold setting for the immunofluorescence analysis for image analysis protocol 2	70
Figure 3.1: Experimental timeline for the study of unilateral AAV5-CBA human WT alpha-synuclein delivery to the rat substantia nigra on motor performance of rats over 12 weeks post-AAV delivery	80
Figure 3.2: Experimental timeline for the study of unilateral AAV5-CBA human WT alpha-synuclein delivery to the rat substantia nigra on motor performance of rats over 16 weeks post-AAV delivery	81
Figure 3.3: Alpha-synuclein immunofluorescence in the substantia nigra of the un-injected and injected hemispheres of the AAV-WT alpha-synuclein group at 12 weeks post-AAV delivery	85
Figure 3.4: GFP immunofluorescence in the substantia nigra of the un-injected and injected hemispheres of the AAV-eGFP group at 12 weeks post-AAV delivery	86
Figure 3.5: Percentage success and number of pellets retrieved in the staircase test prior to AAV-delivery and at multiple time points post-AAV delivery.	88
Figure 3.6: Performance in the cylinder test at nine weeks post-AAV delivery.	90
Figure 3.7: Decreased rears were identified over the 5 minute testing period pre-AAV delivery and at nine weeks post-AAV delivery.	91
Figure 3.8: Performance in the cylinder test at 11 weeks post-AAV delivery	93
Figure 3.9: Performance in the cylinder test at 12 weeks post-AAV delivery	95
Figure 3.10: Limb use asymmetry across multiple timepoints in the cylinder test	96
Figure 3.11: Alpha-synuclein immunofluorescence in the substantia nigra of the un-injected and injected hemispheres of the AAV-WT alpha-synuclein group at 16 weeks post-AAV delivery with both sexes combined	99
Figure 3.12: Alpha-synuclein immunofluorescence in the substantia nigra of the un-injected and injected hemispheres of the AAV-WT alpha-synuclein group at 16 weeks post-AAV delivery for males (a) and females (b) separately	100
Figure 3.13: Number of pellets retrieved and percentage success at six weeks post-AAV delivery in the staircase test	102
Figure 3.14: Number of pellets retrieved and percentage success at nine weeks post-AAV delivery in the staircase test	104
Figure 3.15: Number of pellets retrieved and percentage success at 12 weeks post-AAV delivery in the staircase test	106
Figure 3.16: Number of pellets retrieved and percentage success at 14 weeks post-AAV delivery in the staircase test	108

Figure 3.17: Performance in the cylinder test at 14 weeks post-AAV delivery when both sexes were combined	110
Figure 3.18: Performance in the cylinder test at 14 weeks post-AAV delivery for males alone	112
Figure 3.19: Performance in the cylinder test at 14 weeks post-AAV delivery for females alone	114
Figure 3.20: Limb use asymmetry in the cylinder test pre-AAV delivery and 14 weeks post-AAV delivery	115
Figure 3.21: Total rotations 15 weeks post-AAV delivery in the amphetamine test	116
Figure 3.22: Ipsilateral rotations 15 weeks post-AAV delivery in the amphetamine test	117
Figure 3.23: Number of ipsilateral rotations at 15 weeks post-AAV delivery 5-30 minutes post-amphetamine administration in the amphetamine induced rotation test	119
Figure 3.24: Number of ipsilateral rotations at 15 weeks post-AAV delivery in the amphetamine induced rotation test 31-60 minutes post-amphetamine administration	120
Figure 3.25: Distance travelled in the open field test pre-AAV delivery and 13 weeks post-AAV delivery	121
Figure 4.1: Experimental timeline for the in vivo phase of the 3.5 week AAV-A53T alpha-synuclein study including behavioural testing timepoints, treatment groups, and tissue collection timepoint	134
Figure 4.2: Experimental timeline for the in vivo phase of the 6.5 week AAV-A53T alpha-synuclein study including behavioural testing timepoints, treatment groups, and tissue collection timepoint	135
Figure 4.3: Experimental timeline for the in vivo phase of the 13 week AAV-A53T alpha-synuclein study including behavioural testing timepoints, treatment groups, and tissue collection timepoint	136
Figure 4.4: Illustration of the modifications made to the cylinder test between the 6.5 and 13 week AAV-A53T studies	140
Figure 4.5: Illustration of the modifications made to the amphetamine rotation test between the 22 days post-AAV delivery timepoint and the subsequent testing in the amphetamine rotation test	142
Figure 4.6: Comparison of the injected and un-injected hemispheres of the substantia nigra of the animals in the AAV-A53T group	144
Figure 4.7: Representative images of alpha-synuclein and TH immunofluorescence in the substantia nigra 3.5 weeks post-delivery	146
Figure 4.8: Performance in the cylinder test 25 days post-AAV delivery for both sexes combined	148
Figure 4.9: Limb use asymmetry in the cylinder test at baseline and 25 days post-AAV delivery	150
Figure 4.10: Performance in the stepping test at 24 days post-AAV delivery for both sexes combined	152
Figure 4.11: Performance in the stepping test at 24 days post-AAV delivery for males alone	154
Figure 4.12: Performance in the stepping test at 24 days post-AAV delivery for females alone	155
Figure 4.13: Number of pellets retrieved and percentage success at 21-23 days post-AAV delivery in the staircase test	157
Figure 4.14: Analysis of TH immunoreactive cell counts in the substantia nigra expressed as a percentage of the un-injected side	159
Figure 4.15: Representative images of TH immunoreactivity in the substantia nigra at 3.5 weeks post-AAV delivery	160
Figure 4.16: Representative images TH immunoreactivity in the substantia nigra at 3.5 weeks post-AAV delivery	161
Figure 4.17: Comparison of immunofluorescence intensity in the injected and un-injected hemispheres of the substantia nigra of the animals in the AAV-A53T group at 6.5 weeks post-AAV delivery	163

Figure 4.18: Comparison of the immunofluorescence intensity in the substantia nigra of alpha-synuclein when expressed as a percentage of the un-injected side for the AAV-empty and AAV-A53T groups at 6.5 weeks post-AAV delivery	164
Figure 4.19: Representative images of alpha-synuclein and TH immunofluorescence in the substantia nigra 6.5 weeks post-AAV delivery	167
Figure 4.20: Number of pellets retrieved and percentage success at 23 days post-AAV delivery in the staircase test	170
Figure 4.21: Number of pellets retrieved and percentage success rate at 43 days post-AAV delivery in the staircase test	172
Figure 4.22: Performance in the cylinder test at 44 days post-AAV delivery	174
Figure 4.23: Performance in the cylinder test 44 days post-AAV delivery for males alone	176
Figure 4.24: Performance in the cylinder test 44 days post-AAV delivery for females alone	178
Figure 4.25: Limb use asymmetry 44 days post-AAV delivery in the cylinder test	179
Figure 4.26: Performance in the stepping test at 24 days post-AAV delivery for both sexes combined	181
Figure 4.27: Performance in the stepping test 24 days post-AAV delivery for males alone	183
Figure 4.28: Performance in the stepping test 24 days post-AAV delivery for females alone	185
Figure 4.29: Performance in the stepping test 42 days post-AAV delivery for both sexes combined	187
Figure 4.30: Performance in the stepping test 42 days post-AAV delivery for males alone	189
Figure 4.31: Performance in the stepping test 42 days post-AAV delivery for females alone	191
Figure 4.32: Analysis of TH immunoreactive cell counts in the substantia nigra expressed as a percentage of the un-injected side for both sexes combined	192
Figure 4.33: Representative images of TH immunoreactivity in the substantia nigra at 6.5 weeks post-AAV delivery	193
Figure 4.34: Comparison of alpha-synuclein immunofluorescence in the substantia nigra in the injected and un-injected hemispheres of the animals in the AAV-A53T group at 13 weeks post-AAV delivery	195
Figure 4.35: Comparison of alpha-synuclein immunofluorescence in the substantia nigra when expressed as a percentage of the un-injected side for the AAV-empty and AAV-A53T groups	196
Figure 4.36: Representative images of alpha-synuclein and TH immunofluorescence in the substantia nigra 13 weeks post-AAV delivery	199
Figure 4.37: Number of pellets retrieved at 30-33 days post-AAV delivery in the staircase test	202
Figure 4.38: Number of pellets retrieved at 48-51 days post-AAV delivery in the staircase test	203
Figure 4.39: Number of pellets retrieved on the ipsilateral and contralateral sides at 61-64 days post-AAV delivery in the staircase test	205
Figure 4.40: Number of pellets retrieved and percentage success at 76-78 days post-AAV delivery in the staircase test	207
Figure 4.41: Number of pellets retrieved and percentage success at 83 days post-AAV delivery in the staircase test	209
Figure 4.42: Performance in the cylinder test at 50-52 days post-AAV delivery for both sexes combined	211
Figure 4.43: Performance in the cylinder test at 50-52 days post-AAV delivery for males alone	213
Figure 4.44: Performance in the cylinder test at 50-52 days post-AAV delivery for females alone	215
Figure 4.45: Limb use asymmetry 50-52 days post-AAV delivery in the cylinder test	216
Figure 4.46: Limb use asymmetry for wall at 50-52 days post-AAV delivery in the cylinder test	217

Figure 4.47: Limb use asymmetry for floor at 50-52 days post-AAV delivery in the cylinder test	218
Figure 4.48: Performance in the cylinder test at 86 days post-AAV delivery for both sexes combined	220
Figure 4.49: Performance in the cylinder test at 86 days post-AAV delivery for males alone	222
Figure 4.50: Performance in the cylinder test at 86 days post-AAV delivery for females alone	224
Figure 4.51: Limb use asymmetry 86 days post-AAV delivery in the cylinder test	226
Figure 4.52: Limb use asymmetry 86 days post-AAV delivery in the cylinder test for wall alone	226
Figure 4.53: Limb use asymmetry 86 days post-AAV delivery in the cylinder test for floor alone	227
Figure 4.54: Performance in the stepping test at 84 days post-AAV delivery for both sexes combined	230
Figure 4.55: Performance in the stepping test for males alone at 84 days post-AAV delivery	232
Figure 4.56: Performance in the stepping test for females alone at 84 days post-AAV delivery	234
Figure 4.57: Total number of rotations at 22 days post-AAV delivery	235
Figure 4.58: Number of ipsilateral rotations at 22 days post-AAV delivery	236
Figure 4.59: Number of contralateral rotations at 22 days post-AAV delivery	237
Figure 4.60: Total number of rotations per minute at 50-52 days post-AAV delivery	239
Figure 4.61: Number of ipsilateral rotations per minute at 50-52 days post-AAV delivery	240
Figure 4.62: Number of contralateral rotations per minute at 50-52 days post-AAV delivery	241
Figure 4.63: Total number of rotations at 86 days post-AAV delivery	242
Figure 4.64: Number of ipsilateral rotations at 86 days post-AAV delivery	243
Figure 4.65: Number of contralateral rotations at 86 days post-AAV delivery	244
Figure 4.66: Analysis of TH immunoreactive cell counts in the substantia nigra as expressed as a percentage of the un-injected side	246
Figure 4.67: Representative images of TH immunoreactivity in the substantia nigra at 13 weeks post-AAV delivery	247
Figure 5.1: Experimental timeline for the in vivo phase including behavioural testing timepoints, treatment groups, and tissue collection timepoint	263
Figure 5.2: Exposure to the staircase test for 10 sessions of training in order to improve baseline performance	266
Figure 5.3: Comparison of the automated scoring of the rotational behaviour with subjective scoring of the recordings by an observer	268
Figure 5.4: The total number of ipsilateral rotations as observed over a 120 minute period	269
Figure 5.5: Representative images of alpha-synuclein and tyrosine hydroxylase immunofluorescence in the substantia nigra six weeks post-PFF delivery	272
Figure 5.6: Representative images of alpha-synuclein and tyrosine hydroxylase immunofluorescence in the substantia nigra six weeks post-PFF delivery	273
Figure 5.7: Representative images of alpha-synuclein and tyrosine hydroxylase immunofluorescence in the substantia nigra six weeks post-AAV PFF delivery	274
Figure 5.8: Representative images of alpha-synuclein and tyrosine hydroxylase immunofluorescence in the substantia nigra six weeks post-AAV PFF delivery	275
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	276
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	278
Figure 5.11: Performance in the cylinder test four weeks post-AAV PFF delivery with both sexes combined	280

Figure 5.12: Performance in the cylinder test four weeks post-AAV PFF delivery for males alone	282
Figure 5.13: Performance in the cylinder test four weeks post-AAV PFF delivery for females alone	284
Figure 5.14: Limb use asymmetry in the cylinder test at baseline and four weeks post-AAV PFF delivery	286
Figure 5.15: Performance in the stepping test at three weeks post-AAV PFF delivery	288
Figure 5.16: Performance in the stepping test three weeks post-AAV PFF delivery for males alone	290
Figure 5.17: Performance in the stepping test at three weeks post-AAV PFF delivery for females alone	292
Figure 5.18: Performance in the stepping test at 4.5 weeks post-AAV PFF delivery	294
Figure 5.19: Performance in the stepping test four weeks post-AAV PFF delivery for males alone	296
Figure 5.20: Performance in the Stepping Test at 4.5 weeks post-AAV PFF delivery for females alone	298
Figure 5.21: Total number of rotations at five weeks post-AAV PFF delivery	300
Figure 5.22: Number of ipsilateral rotations at five weeks post-AAV PFF delivery	301
Figure 5.23: Number of contralateral rotations at five weeks post-AAV PFF delivery	302
Figure 5.24: Number of pellets retrieved two weeks post-AAV PFF delivery in the staircase test	304
Figure 5.25: Percentage success and number of pellets retrieved at four weeks post-AAV PFF delivery in the staircase test	306
Figure 5.26: Representative images of tyrosine hydroxylase immunohistochemistry in the substantia nigra	308
Figure 5.27: Representative images of tyrosine hydroxylase immunohistochemistry in the substantia nigra	309
Figure 5.28: Representative images of tyrosine hydroxylase immunohistochemistry in the substantia nigra	310
Figure 5.29: Tyrosine hydroxylase immunoreactive cells expressed as a percentage of the un-injected side six weeks post-AAV PFF delivery	311
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	312
Figure 6.1: Summary of the main findings of this thesis.	323
Figure 6.2: The signalling and functions of 17 β -estradiol	331
Figure I.1: Performance in the cylinder test pre-AAV delivery	338
Figure I.2: Number of pellets retrieved and percentage success at baseline, prior to AAV delivery in the staircase test	340
Figure I.3: Performance in the cylinder test pre-AAV delivery for both sexes combined	342
Figure I.4: Performance in the cylinder test at for males alone pre-AAV delivery	344
Figure I.5: Performance in the cylinder test at for females alone pre-AAV delivery	346
Figure II.1: Baseline performance in the cylinder test for both sexes combined	348
Figure II.2: Baseline performance in the stepping test for both sexes combined	350
Figure II.3: Baseline performance in the stepping test for males alone	352
Figure II.4: Baseline performance in the stepping test for females alone	353
Figure II.5: Number of pellets retrieved and percentage success at baseline testing in the staircase test	355
Figure II.6: Number of pellets retrieved and percentage success at baseline testing in the staircase test	357
Figure II.7: Baseline performance in the stepping test for both sexes combined	359
Figure II.8: Baseline performance in the stepping test for males alone	360
Figure II.9: Baseline performance in the stepping test for females alone	362
Figure II.10: Number of pellets retrieved and percentage success at baseline testing in the staircase test	364
Figure II.11: Performance in the cylinder test at 50-52 days post-AAV delivery for both sexes combined	366

Figure II.12: Performance in the cylinder test at 50-52 days post-AAV delivery for males alone	368
Figure II.13: Performance in the cylinder test at 50-52 days post-AAV delivery for females alone	370
Figure II.14: Performance in the cylinder test at 86 days post-AAV delivery for both sexes combined	372
Figure II.15: Performance in the cylinder test at 86 days post-AAV delivery for males alone	374
Figure II.16: Performance in the cylinder test at 86 days post-AAV delivery for females alone	376
Figure II.17: Baseline performance in the stepping test for both sexes combined	378
Figure II.18: Baseline performance in the stepping test for males alone	380
Figure II.19: Baseline performance in the stepping test for females alone	382
Figure II.20: Total number of rotations per minute at 22 days post-AAV delivery	383
Figure II.21: Number of ipsilateral rotations per minute at 22 days post-AAV delivery	384
Figure II.22: Number of contralateral rotations per minute at 22 days post-AAV delivery	385
Figure II.23: Number of ipsilateral rotations per minute at 86 days post-AAV delivery	386
Figure II.24: Number of contralateral rotations per minute at 86 days post-AAV delivery	387
Figure III.1: Baseline performance in the cylinder test	389
Figure III.2: Baseline performance in the cylinder test for males alone	391
Figure III.3: Baseline performance in the cylinder test for females alone	393
Figure III.4: Baseline performance in the stepping test with both sexes combined	395
Figure III.5: Baseline performance in the stepping test for males alone	397
Figure III.6: Baseline performance in the stepping test for females alone	399
Figure III.7: Percentage success and number of pellets retrieved at baseline testing in the staircase test	401

List of Tables

Table 1.1: Examples of chemically induced and genetic models of PD.	29
Table 1.2: Studies using AAV expression of WT alpha-synuclein in rats	36
Table 1.3: Studies using AAV expression of A53T alpha-synuclein in rats	37
Table 1.4: Adeno-associated viral vector serotypes, including known receptors and CNS cell tropism.	39
Table 2.1: Criteria for scoring of the staircase test.	57
Table 2.2: Exclusion criteria for the staircase test.	57
Table 2.3: Blocking agents and antibody dilutions for free-floating immunohistochemistry.	65
Table 2.4: Blocking agents and antibody dilutions for free-floating immunohistochemistry.	66
Table 2.5: Blocking agents and antibody dilutions for free-floating immunofluorescence.	67
Table 2.6: Blocking agents and antibody dilutions for immunofluorescence.	68
Table 3.1: Details of the treatment groups (Virus Titre, Injection Volumes and Coordinates) for the study of unilateral AAV5-CBA human WT alpha-synuclein delivery to the rat substantia nigra, over 12 and 16 weeks post-AAV delivery.	82
Table 3.2: A summary of the results obtained in the 12 week AAV-WT study.	98
Table 3.3: A summary of the results obtained in the 16 week AAV-WT study.	122
Table 4.1: Virus Titre, Injection Volumes and Coordinates for the A53T studies.	137
Table 4.2: Analysis of alpha-synuclein immunofluorescence in the substantia nigra in the injected and un-injected hemisphere of animals in the AAV-alpha-synuclein group.	145
Table 4.3: A summary of the results obtained in the 3.5 week AAV-A53T study.	162
Table 4.4: Analysis of alpha-synuclein immunofluorescence in the substantia nigra in the injected and un-injected hemisphere of animals in the AAV-A53T alpha-synuclein and AAV-empty groups	166
Table 4.5: Descriptive statistics for alpha-synuclein expression as a percentage of the un-injected side in the AAV-empty and AAV-A53T groups in the substantia nigra	168
Table 4.6: A summary of the results obtained in the 6.5 week AAV-A53T study.	194
Table 4.7: Analysis of alpha-synuclein immunofluorescence in the injected and un-injected hemisphere of the substantia nigra of animals in the AAV-A53T alpha-synuclein and AAV-empty groups	198
Table 4.8: Descriptive statistics for alpha-synuclein immunofluorescence in the substantia nigra as a percentage of the un-injected side in the AAV-empty and AAV-A53T groups	200
Table 4.9: A summary of the results obtained in the 13 week AAV-A53T study.	248
Table 5.1: Virus Titre, Virus and PFF Injection Volumes and Coordinates	264
Table 5.2: Visible alpha-synuclein immunofluorescence across the substantia nigra in the alpha-synuclein treatment groups at six weeks post-AAV PFF delivery.	271
Table 5.3: Summary of behavioural results.	313
Table 5.4: Summary of tyrosine hydroxylase immunohistochemistry results.	314

List of Abbreviations

6-OHDA	6-hydroxydopamine
AAV	adeno-associated viral
AD	Alzheimer's disease
ANOVA	analysis of variance
AP	anterior-posterior
APP	amyloid precursor protein
ATP	adenosine triphosphate
AU	arbitrary units
Aβ	amyloid β
BAC-Tg	bacterial artificial chromosome
BGH	Bovine Growth Hormone
CAG	chicken beta-actin cytomegalovirus hybrid promoter
cAMP	cyclic adenosine monophosphate
CBA	chicken beta actin
CCL2	CC-chemokine ligand 2
CCL7	CC-chemokine ligand 7
CMA	chaperone mediated autophagy
CMV	cytomegalovirus
CNS	central nervous system
DAB	3,3-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DAT	dopamine transporter
DLB	Dementia with Lewy bodies
DNA	deoxyribonucleic acid
DRP-1	dynamin related protein 1
DV	dorsal-ventral
eGFP	enhanced Green Fluorescent Protein
ELISA	enzyme-linked immunosorbent assay
ENS	enteric nervous system
EPM	elevated plus maze
ER	endoplasmic reticulum

ERalpha	estrogen receptor alpha
ERbeta	estrogen receptor beta
ERK1/2	extracellular signal–regulated kinases 1/2
EtOH	Ethanol
FST	forced swim test
GABA	gamma-aminobutyric acid
GBA1	Glucosylceramidase Beta 1
Gcas	glucocerebrosidase
GFP	Green Fluorescent Protein
GPR30	G protein-coupled receptor 30
GWAS	genome-wide association studies
H2O2	hydrogen peroxide
HCl	Hydrochloric Acid
IL-1beta	Interleukin 1beta
IL6	Interleukin 6
iNOS	inducible nitric oxide synthase
iPSCs	induced pluripotent stem cells
ITRs	inverted terminal repeats
JAK-STAT	janus kinase and signal transducer and activator of transcription
JAK1/2	janus kinase 1/2
L-DOPA	L-3,4-dihydroxyphenylalanine
LAMP2	lysosome-associated membrane protein 2
LBD	Lewy Body Dementia
LPS	lipopolysaccharide
LRRK2	leucine rich repeat kinase 2
MAM	mitochondria-associated-membranes
MeOH	methanol
ML	medial-lateral
MPP+	1-methyl-4-phenylpyridium
mPrP	mThy-1 mouse prion promoter
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
MSA	multiple systems atrophy

NF-κB	nuclear factor kappa B
NFAT	nuclear factor of activated T-cells
NLRP3	NLR family pyrin domain containing 3
NSAIDs	non-steroidal anti-inflammatory drugs
PBS	Phosphate Buffered Saline
PD	Parkinson's disease
PDGF-B	platelet derived growth factor B-chain
PFA	paraformaldehyde
PFFs	preformed fibrils
PINK1	phosphatase and tensin homologue-induced kinase 1
PLK2	polo-like kinase 2
PTEN	phosphatase and tensin homologue
REM	rapid eye movement
rtPCR	real-time polymerase chain reaction
SEM	standard error of the mean
siRNA	small interfering ribonucleic acid
SNARE	soluble NSF attachment protein receptor
ssDNA	single strand DNA
TFEB	transcription factor EB
TH	tyrosine hydroxylase
TLR2	toll-like receptor 2
TLR4	toll-like receptor 4
TNF	Tumor Necrosis Factor
TNF-α	tumor necrosis factor-alpha
TRIM28	tripartite motif-containing 28
UPR	unfolded protein response
VAMP2	synaptobrevin-2/vesicle-associated membrane protein
VMAT2	vesicular monoamine transporter 2
WPRE	woodchuck hepatitis virus post-transcriptional control element
WT	wildtype

Abstract

Parkinson's disease (PD) is the most common motor neurodegenerative disease, characterised histologically by the presence of Lewy bodies, which are intracellular inclusions containing a variety of cellular proteins, the most abundant of which is a protein called alpha-synuclein. Autosomal dominant forms of PD can include N-terminal point mutations in alpha-synuclein such as A53T, as well as genomic duplications or triplications that contain the alpha-synuclein locus. The development of animal models associated with the over-expression of alpha-synuclein, including its mutated forms, are essential to gain insights into disease mechanisms and in drug development efforts which aim to slow the progression or cure the disease. This study aimed to develop and characterise a humanised model of PD associated with delivery of adeno-associated virus (AAV) expressing alpha synuclein and pre-formed alpha-synuclein fibrils (PFFs) unilaterally into the rat substantia nigra. Rats were unilaterally injected with a single injection of AAV5-CBA-alpha-synuclein wildtype (WT) human, AAV1/2-CMV-A53T human alpha-synuclein, PFF WT alpha-synuclein human, PFF A53T alpha-synuclein human, or two injections, one containing AAV and one containing PFF, into the substantia nigra and assessed at multiple time-points post-delivery for changes in motor behaviours in the stepping, cylinder, staircase and amphetamine rotation tests. Tyrosine hydroxylase (TH) immunoreactive cell loss was assessed through the use of TH immunohistochemistry to quantify dopamine neuronal cell loss. The presence of alpha-synuclein within the substantia nigra was verified by immunofluorescent staining of post-mortem tissue sections. The combination of AAV + PFF A53T injection into the male rat substantia nigra was found to be effective at stimulating motor deficits in the stepping test, with decreased contralateral stepping observed when compared with their ipsilateral stepping, as well as in the cylinder test, where increased ipsilateral wall contacts were observed when compared to all other treatment groups, and in the amphetamine test, where increased ipsilateral rotations were observed when compared to all other treatment groups. Furthermore neuronal cell loss, expressed as a percentage of the un-injected hemisphere, was observed in the

substantia nigra of the PFF + AAV A53T treatment group when compared with PFF A53T alpha-synuclein treatment group at 6 weeks post-AAV + PFF delivery when all alpha-synuclein treated groups were compared. When the same treatment was given to female rats the development of motor behaviours in the stepping test occurred at 4.5 weeks post-AAV + PFF delivery, which is later than that observed in the male rats, at 3 weeks post-AAV delivery, indicating a less severe and/or more slowly progressive phenotype compared to males. Intranigral injection of AAV1/2-CMV-A53T human alpha-synuclein led to the loss of TH immunoreactive cells in the absence of a consistent motor deficit up to 13 weeks post-delivery, while intranigral injection of AAV5-CBA-alpha-synuclein WT did not lead to the development of motor deficits or TH immunoreactive cell loss up to 16 weeks post-delivery. The results of this research provide support for use of a combination of AAV + PFF mediated expression of A53T alpha-synuclein in the male rat substantia nigra as a preclinical model of synucleinopathy. The addition of the alpha-synuclein PFFs with the AAV alpha-synuclein is likely to lead to a greater accumulation of alpha-synuclein, potentially through the seeding of the PFFs and the recruitment of endogenous alpha-synuclein as a result. The development of a motor phenotype in addition to dopamine neuronal cell loss over a 6 week period lends well to the potential of the model for screening drug candidates which may slow the progression or protect against synuclein-induced neurodegeneration. However, this is a supraphysiological model of synuclein induced pathology and it remains to be confirmed that such a rapid induction of synuclein induced neurodegeneration bears a close resemblance to the human condition.

Summary

Parkinson's disease (PD) is the most common motor neurodegenerative disease, characterised histologically by the presence of Lewy bodies, which are intracellular inclusions containing a variety of cellular proteins, the most abundant of which is a protein called alpha-synuclein. Clinically, a diagnosis of PD is made based upon the emergence of the cardinal motor features associated with the disease, which include bradykinesia, postural instability, rigidity of the limbs and resting tremor. The most common pharmacological treatment for PD is centred around replenishing the decreased levels of dopamine, through the administration of levodopa in combination with a dopa decarboxylase inhibitor. While this treatment shows a good response initially, the effectiveness of this treatment decreases over time and dyskinesias have been found to develop as a side effect. The aetiology of PD remains unclear, however it has been suggested that a complex interaction between environment and genetics can lead to the development of the disease. Most cases of PD are idiopathic in nature, with approximately 10-20% of cases having a family history of the disease. Many genetic causes have been identified for the development of PD since the initial discovery of the mutation in alpha-synuclein in 1997 including, but not limited to, mutations in leucine rich repeat kinase 2 (LRRK2) which leads to the development of an autosomal dominant form of PD, DJ-1 and parkin, both of which lead to the development of autosomal recessive forms of the disease. Autosomal dominant forms of PD can also be caused by N-terminal point mutations in alpha-synuclein such as A53T, A30P, E46K, G51D, A53E and H50Q, as well as genomic duplications or triplications that contain the alpha-synuclein locus.

Much research has been performed in order to develop a preclinical model of PD, including toxin based models such as 6-OHDA and MPTP, and transgenic models utilising alpha-synuclein transgene expression. While these models have proven useful in both the testing of novel compounds to treat disease progression and the development of a better understanding of some of the pathological mechanisms underlying disease progression, they are not without

their limitations, which include a lack of translation to the human condition and compensatory mechanisms masking the development of pathology. With this in mind, the present study aimed to develop a more translatable model of synucleinopathy through the use of adeno-associated viral (AAV) technology and preformed fibrils of alpha-synuclein (PFFs), delivered to the rat substantia nigra.

Rats were unilaterally injected with a single injection of AAV5-CBA-alpha-synuclein wildtype (WT) human, AAV1/2-CMV-A53T human alpha-synuclein, PFF WT alpha-synuclein human, PFF A53T alpha-synuclein (human), or two injections, one containing AAV and one containing PFF, into the substantia nigra and assessed at multiple time-points post-delivery for changes in motor behaviours and TH immunoreactive cell loss. It was found that a combination of AAV + PFF A53T injection into the male rat substantia nigra was most effective at replicating the common motor deficits and neuronal cell loss associated with preclinical models of PD within a 6 week timeframe. More specifically, in the stepping test decreased contralateral stepping were observed when compared with their ipsilateral stepping, while in the cylinder test increased ipsilateral wall contacts were observed when compared to all other treatment groups, and finally, in the amphetamine test, where increased ipsilateral rotations were observed when compared to all other treatment groups. Furthermore neuronal cell loss, expressed as a percentage of the un-injected hemisphere, was observed in the substantia nigra of the PFF + AAV A53T treatment group when compared with PFF A53T alpha-synuclein treatment group at 6 weeks post-AAV + PFF delivery when all alpha-synuclein treated groups were compared. When the same treatment was given to female rats the development of motor behaviours occurred at a later time point than that observed in the male rats. Specifically, contralateral stepping deficits in the stepping test were observed at 4.5 weeks post-AAV PFF delivery in the females, while these deficits were observed in the males at 3 weeks post-AAV PFF delivery. Intranigral injection of AAV1/2-CMV-A53T human alpha-synuclein led to the loss TH immunoreactive cells in the absence of a consistent motor deficit up to 13 weeks post-delivery, while intranigral injection of AAV5-CBA-alpha-

synuclein WT did not lead to the development of motor deficits or TH immunoreactive cell loss up to 16 weeks post-delivery. The results of this research provide support for use of a combination of AAV + PFF mediated expression of A53T alpha-synuclein in the male rat substantia nigra as a preclinical model of synucleinopathy, although further research will be required to confirm this models utility as a translatable model of synucleinopathy.

1. Introduction

1.1 Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease and the most common motor neurodegenerative disease in the world (Burke and O'Malley, 2013). The main risk factor for the development of PD is increasing age, with a large increase in the prevalence of the disease in persons over 50 years of age (Bower et al., 2000, Driver et al., 2009). Due to the increasing life expectancy observed in many countries, it is expected that the prevalence of PD will double as the next generation ages (Driver et al., 2009).

The cardinal features of PD include bradykinesia, postural instability, rigidity of the limbs and resting tremor (Lees et al., 2009). While PD is diagnosed clinically, a definitive diagnosis cannot be made until the patient has died, at which point the motor symptoms observed while the patient was alive can be combined with the post-mortem assessment of the loss of dopaminergic neurons in the substantia nigra and the presence of Lewy bodies. Lewy bodies are intracellular inclusions containing a variety of cellular proteins, the most abundant of which is a protein called alpha-synuclein (Burke and O'Malley, 2013). In a healthy brain the dopaminergic cells of the substantia nigra project rostrally through the medial forebrain bundle to the striatum, where they release dopamine. Due to the loss of these dopaminergic cells in PD, this release of dopamine is greatly reduced, leading to the development of motor symptoms.

As well as the motor symptoms of PD, there are many prodromal symptoms which can manifest before the motor symptoms. Early symptoms of the development of PD include symptoms which demonstrate autonomic dysfunction, including gastrointestinal and sexual dysfunction, urinary disturbance, cardiovascular dysregulation, pupillo-motor and tear abnormalities, and thermoregulatory aberrance (Merola et al., 2018). Symptoms of olfactory dysfunction such as decreased odour detection,

impaired odour identification, and a reduction in olfactory bulb volume, have been found to present in approximately 80% of PD patients (Chen et al., 2015a). Other non-motor symptoms which have been found to occur in PD include sleep disorders, cognitive deficits and mood disorders. These symptoms are thought to reflect other neurotransmitter abnormalities, including abnormalities in serotonin, noradrenaline, acetylcholine and gamma-aminobutyric acid (GABA) (Creed et al., 2019).

It has been estimated that in order for PD motor symptoms to develop there needs to be an approximately 30% loss of dopaminergic neurons in the substantia nigra, with a 50-60% dopaminergic depletion in the striatum (Fearnley and Lees, 1991, Ma et al., 1997, Greffard et al., 2006). This is in contrast to the more prevailing idea that at the time of diagnosis most PD patients have had a loss of approximately 60% of their substantia nigra dopaminergic neurons (Dauer and Przedborski, 2003), and a depletion of 80% of the dopaminergic content of the striatum (Dauer and Przedborski, 2003, Kish et al., 1988). This suggests that perhaps there is a timeframe following onset of disease where a large number of the remaining dopaminergic neurons could be rescued (Burke and O'Malley, 2013).

While the nigro-striatal tract is the main region associated with the motor symptoms of PD, there are numerous other regions which are associated with the development of the non-motor symptoms of PD, including the locus coeruleus, cerebellum, thalamus, hypothalamus, olfactory bulb, limbic system and brainstem. Locus coeruleus cell loss has been hypothesised to lead to increased dopaminergic degeneration in the substantia nigra, with noradrenergic hyperinnervation leading to neuroprotective effects (Henrich et al., 2018).

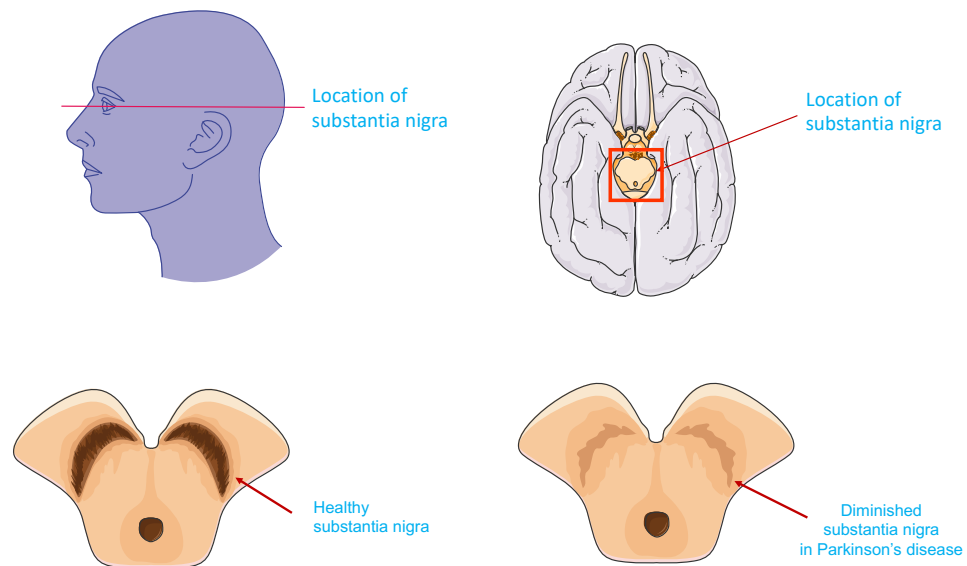


Figure 1.1: The location of the substantia nigra in the human brain, with the neuromelanin pigmented substantia nigra defined as the healthy substantia nigra, and the neuromelanin diminished substantia nigra representative of dopaminergic cell loss in PD.

1.1.1 Treatments for Parkinson's disease

The most common pharmacological treatment for PD is centred around replenishing the decreased levels of dopamine, through the administration of levodopa in combination with a dopa decarboxylase inhibitor. While this treatment shows a good response initially, the effectiveness of this treatment decreases over time and dyskinesias have been found to develop as a side effect (Bastide et al., 2015). Other treatments for PD include dopamine agonists, monoamine oxidase B inhibitors, anticholinergic agents, and amantadine which can be used to treat the dyskinesias induced by prolonged levodopa treatment. While the treatments available are capable of providing some symptomatic relief from the motor symptoms associated with the disease, there are a number of other symptoms for which there are limited, or no treatment, including cognitive decline.

While there have been advances in the treatment of PD in recent years, including the development of deep brain stimulation, all existing therapies provide only symptomatic relief, with no treatments available to halt or slow disease progression (Lees et al., 2009). Deep brain stimulation has proved

efficacious, however there are many risks associated with the treatment due to its invasive nature, including cognitive, speech and mood impairments. Furthermore, many clinical trials have failed, including the trial of CEP-1347, an inhibitor of mixed lineage kinases involved in the activation of apoptotic pathways implicated in PD, in the PRECEPT trial (Burke and O'Malley, 2013). Finally, attempts at grafting embryonic mesencephalic neurons into the striatum of PD patients found that these patients still develop Lewy bodies some years after grafting (Kordower et al., 2008, Li et al., 2008).

1.1.2 Environmental Toxins and their impact on Parkinson's disease pathology

The aetiology of PD remains unclear, however it has been suggested that a complex interaction between environment and genetics can lead to the development of the disease. Most cases of PD are idiopathic in nature, with approximately 10-20% of cases having a family history of the disease (Deng et al., 2018). Exposure to a variety of elements such as industrial chemicals, heavy metals, manganese mines, pesticides and herbicides have all been implicated as risk factors for the development of PD (Gao and Hong, 2011, de Lau and Breteler, 2006). Furthermore, epidemiological studies have linked living in rural areas, drinking well water, brain trauma and early life inflammation as risk factors for the development of PD (Gao and Hong, 2011).

Paraquat is a widely used herbicide which has been found to lead to the development of motor symptoms and nigrostriatal degeneration when administered to mice (McCormack et al., 2002). These findings have not been consistently replicated (Cicchetti et al., 2005), however they do provide support for the impact of environmental toxins on the development of PD. Furthermore, rotenone, which is a naturally occurring pesticide, has been linked to PD, as it has the ability to inhibit complex I mitochondrial respiration (Meredith et al., 2008). Systemic administration of rotenone leads to the development of a number of pathological and motor hallmarks of PD, however it has also been found to lead to more widespread cell loss, not just specific to

dopaminergic neurons, with large variations observed in both motor response and animal sensitivity to rotenone (Johnson and Bobrovskaya, 2015).

While the research into the environmental risk factors for the development of PD is compelling, further research is needed in order to untangle the complex gene-environment interactions which may ultimately lead to the development of PD. It is possible that sporadic cases of PD develop in patients predisposed to disease development due to the expression of susceptibility genes.

1.1.3 Parkinson's disease and inflammation

It has been found that people who regularly take non-steroidal anti-inflammatory drugs (NSAIDs) have a decreased propensity to develop PD, and that the use of NSAIDs in PD patients leads to a decreased risk of falls (Chen et al., 2005, Manthripragada et al., 2011). Post-mortem analysis of brains from PD patients have found upregulated levels of pro-inflammatory cytokines, with this increase also evident in the cerebrospinal fluid (Vivekanantham et al., 2015). Tumour necrosis factor-alpha (TNF- α) has been found to induce dopaminergic cell death following its release through microgliosis. Through the targeting of mitochondrial complex-I it leads to the downregulation of adenosine triphosphate (ATP) production which results in increased oxidative stress (Prajapati et al., 2015).

1.1.4 Sex differences in Parkinson's disease

The occurrence of PD is twice as great in males when compared to females (Gillies et al., 2014, Smith and Dahodwala, 2014), with the symptom presentation differing between the sexes. Males present more rigidity and sleep disturbances than females, while females present with increased resting tremor and PD associated depression (Gillies et al., 2014). Furthermore, dopaminergic neurons of the substantia nigra have been found to express the *SRY* gene, which is the sex determining region on the Y sex chromosome, which regulates the expression of dopamine-associated enzymes such as tyrosine hydroxylase (TH) and monoamine oxidase A (Czech et al., 2012).

The difference between the sexes in the development of PD seems to be greatly decreased following the onset of menopause in females (Cerri et al., 2019). Estrogen has been reported to have neuroprotective effects, with 17 β -estradiol associated with protection against PD (Gillies et al., 2014, Smith and Dahodwala, 2014). Pre-lesion administration of 17- β -estradiol has been found to protect against the development of striatal dopaminergic dysfunction in neurotoxin models of PD (Baraka et al., 2011). *In vitro* research has found that estrogen has the ability to prevent alpha-synuclein aggregation and destabilise alpha-synuclein fibrilization (Hirohata et al., 2009). Furthermore, estrogen has been found to reduce pro-inflammatory cytokine production and lead to decreased microglial activation in the brain (Pozzi et al., 2006).

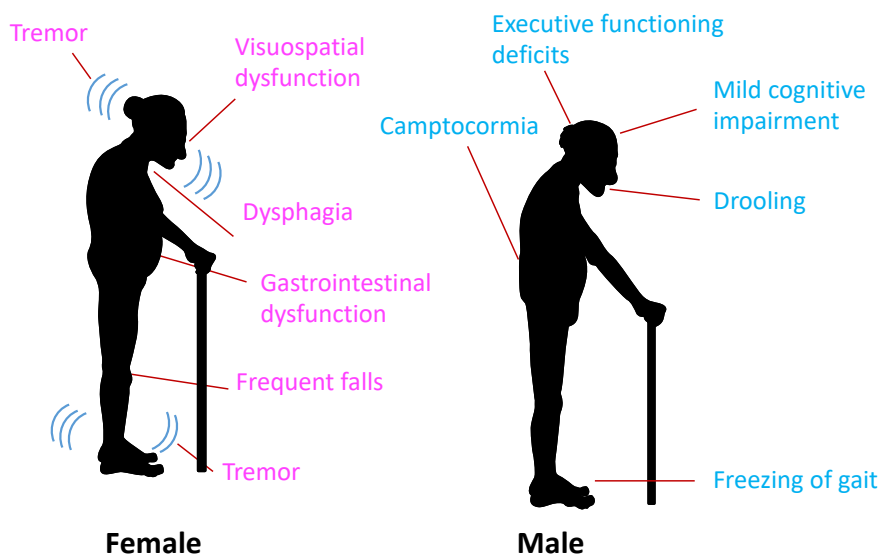


Figure 1.2: Differences in PD symptomatology between males and females. Figure adapted from (Cerri et al., 2019).

1.1.5 Parkinson's disease genetics

Many genetic causes have been identified for the development of PD since the initial discovery of the mutation in alpha-synuclein in 1997 (Polymeropoulos et al., 1997), including, but not limited to, mutations in leucine rich repeat kinase 2 (*LRRK2*) (Paisan-Ruiz et al., 2004, Zimprich et al., 2004) which leads to the development of an autosomal dominant form of PD, *PARKIN* (Kitada et al., 1998), *DJ-1* (Bonifati et al., 2003), and PTEN (phosphatase and tensin homologue)-induced kinase 1 (*PINK1*) (Valente et al., 2004), all of which lead to

the development of autosomal recessive forms of the disease. Mutations in the *PARKIN* gene have been seen in approximately 50% of familial cases of PD (Moore et al., 2005). This gene encodes for a 465-amino-acid protein that functions as an E3 ubiquitin protein ligase which covalently tags proteins with ubiquitin, identifying them for proteasomal degradation by 26S proteins (Moore et al., 2005). *PARKIN* has been found to maintain mitochondrial function and leads to the clearance of dysfunctional mitochondria by autophagy, with loss of function mutations and impaired functioning of *PARKIN* implicated in the prevention of degradation of accumulating pathological proteins (Gao and Hong, 2011). Furthermore, *PARKIN* has been found to become inactivated following exposure to dopaminergic, oxidative and nitrosative stress (Gao and Hong, 2011).

The most common mutated gene identified in PD is *LRRK2*, and it has been identified in both idiopathic and familial forms of disease (Gao and Hong, 2011). In its native state *LRRK2* is hypothesised to play a protective role, leading to the reduction of oxidative stress and the regulation of protein degradation. However, *LRRK2* mutations are associated with cytotoxicity, disrupted kinase activity and impaired mitochondrial function (Gao and Hong, 2011). One of the rarer mutations seen in PD is mutations in *DJ-1*, which is associated with mitochondria, as well as having antioxidant functions (Gao and Hong, 2011).

Autosomal dominant forms of PD can be caused by N-terminal point mutations in alpha-synuclein such as A53T, A30P, E46K, G51D, A53E and H50Q (Polymeropoulos et al., 1997, Kruger et al., 1998, Zarranz et al., 2004, Appel-Cresswell et al., 2013, Proukakis et al., 2013, Lesage et al., 2013, Pasanen et al., 2014). Autosomal dominant forms of the disease can also be caused by genomic duplications or triplications that contain the alpha-synuclein locus (Singleton et al., 2003, Chartier-Harlin et al., 2004). Autosomal dominant forms of PD, due to alpha-synuclein locus duplication, develop at approximately 60 years of age and are known as late onset. In contrast to this, triplication of the alpha-synuclein locus leads to the development of early onset PD, at less than

40 years of age, showing that an increasing alpha-synuclein burden leads to an earlier development of the disease (Singleton et al., 2003).

Alpha-synuclein is also implicated in the development of sporadic PD, with multiple genome-wide association studies (GWAS) identifying single-nucleotide polymorphisms as risk factors which can increase the risk of the development of sporadic PD (Simon-Sanchez et al., 2009, Nalls et al., 2014). One of the single nucleotide polymorphisms which can lead to the development of PD is a risk variant in a noncoding distal enhancer element of *SNCA* which can lead to the development of increased alpha-synuclein expression (Soldner et al., 2016).

1.2 Alpha-synuclein

1.2.1 Alpha-synuclein's physiological role

Alpha-synuclein is a 140-amino-acid protein and consists of three distinct domains: the N-terminal region, containing four imperfect KTKEGV motif repeats that fold into amphipathic helices, which has been found to associate with membranes, particularly the membranes of synaptic vesicles; the hydrophobic non-amyloid component domain which has been found to be crucial for alpha-synuclein aggregation as this region acts as a fibrillization inhibition region; and the C-terminal region which is enriched in highly acidic and charged amino acids, and acts to inhibit protein aggregation (Uversky, 2003, Ozansoy and Basak, 2013). *SNCA*, the gene encoding alpha-synuclein, is located on chromosome 4q22.1. There are six exons present on *SNCA*, five of which are coding regions for alpha-synuclein.

The main physiological functions of alpha-synuclein are not fully understood. Natively, alpha-synuclein has been found to localise to synaptic terminals, where it is found to interact with synaptic vesicles, as well as binding to membranes and inducing membrane curvature (Iwai et al., 1995, Kahle et al., 2000, Davidson et al., 1998, Varkey et al., 2010). It has been found that the soluble NSF attachment protein receptor (SNARE) complex assembly is

regulated by alpha-synuclein (Chandra et al., 2005). Furthermore, through binding synaptobrevin-2/vesicle-associated membrane protein (VAMP2), which is a SNARE protein, alpha-synuclein promotes synaptic vesicle fusion at the presynaptic terminal (Burre et al., 2010).

Alpha-synuclein is also highly expressed in the nucleus (Iwai et al., 1995, Maroteaux et al., 1988), with this expression confirmed by numerous studies (Goncalves and Outeiro, 2013, McLean et al., 2000, Pinho et al., 2019). It has been proposed that through alpha-synucleins localisation in the nucleus it may interact with histones, affecting histone acetylation and thereby modulating gene expression (Goers et al., 2003).

It has been found that alpha-synuclein has the ability to bind to deoxyribonucleic acid (DNA), induce DNA fragmentation, and modulate the DNA repair process (Pinho et al., 2019, Schaser et al., 2019). Alpha-synuclein has also been found to be expressed in mitochondria in the striatum, cortex and substantia nigra (Devi and Anandatheerthavarada, 2010, Li et al., 2007). Peripherally, the presence of alpha-synuclein has also been identified in blood cells, with red blood cells expressing the majority of the alpha-synuclein, however it is also present in peripheral blood mononuclear cells, platelets, and the plasma, with platelets demonstrating the highest levels of alpha-synuclein (Barbour et al., 2008).

1.2.2. Alpha-synuclein conformations

There are many different conformations of alpha-synuclein, including monomeric, tetrameric, oligomeric, and fibrillar as well as aggregates. As an intrinsically disordered protein alpha-synuclein may exist as an unstructured monomer, or following membrane/lipid binding the N-terminal region it may adopt an alpha-helical structure, which promotes membrane curvature, and can lead to the development of other quaternary arrangements, forming tetramers (Bartels et al., 2011, Chandra et al., 2003, Eliezer et al., 2001, Weinreb et al., 1996). It is generally in an acute equilibrium between a soluble

cytosolic form, and an insoluble membrane-bound form (Fortin et al., 2004, Iwai et al., 1995).

1.2.3 Alpha-synuclein and Parkinson's disease

Mutations in alpha-synuclein were identified as the first genetic cause of familial PD, leading to the development of an autosomal dominant form of disease (Polymeropoulos et al., 1997). While the exact causes of neurodegeneration in PD have yet to be elucidated, it is clear that alpha-synuclein is a key player in the pathogenesis of this disease (Poewe et al., 2017). Genomic duplication and triplication of the alpha-synuclein locus, and single nucleotide polymorphisms which lead to the overexpression of WT alpha-synuclein can also lead to the development of disease (Wong and Krainc, 2017).

The A53T point mutation of alpha-synuclein was the first to be described in relation to PD (Polymeropoulos et al., 1997). This mutation is linked to the development of autosomal dominant PD, accelerated alpha-synuclein aggregation, as well as the presence of prominent dementia symptoms (Lesage and Brice, 2009). This mutation occurs at the N-terminal of alpha-synuclein and involves the substitution of an alanine for a threonine. It has been shown to have a high propensity to promote the formation of fibrillar alpha-synuclein, leading in turn to the amplification of protein aggregation (Flagmeier et al., 2016). The increased propensity to aggregate observed in A53T alpha-synuclein when compared to WT has been proposed to be associated with increased beta-sheet formation close to the site of mutation (Coskuner and Wise-Scira, 2013).

During the process of alpha-synuclein aggregation to oligomers, protofibrils and amyloid fibrils, the conformation of alpha-synuclein may change from alpha-helical-rich to beta-sheet-rich (Caughey and Lansbury, 2003). Increased beta-sheet formation can lead to the development of beta-sheet rich insoluble aggregates which can then develop into Lewy bodies. Furthermore, the A53T mutation of alpha-synuclein has shown an increased propensity to misfold (Conway et al., 2000, Zach et al., 2007). Due to the predisposition to aggregate

demonstrated by A53T mutant alpha-synuclein, microglia have been found to adopt a pro-inflammatory state (Sanchez and Maguire-Zeiss, 2020), which may lead to a cascade of neuroinflammatory events such as microgliosis, which may in turn influence the phenotype of other immune cells in the brain (Yun et al., 2018).

Alpha-synuclein has been found to interact with other PD-associated genes. For example, research utilising *LRRK2* overexpression found that this overexpression led to accelerated neuropathological progression in alpha-synuclein A53T mice *tetO-A53T*, with the depletion of *LRRK2* delaying the neuropathological progression (Lin et al., 2009). Furthermore, assessment of the impact of knockout or inhibition of *LRRK2* found that alpha-synuclein-induced neurodegeneration was prevented in these animal models (Daher et al., 2015, Daher et al., 2014). Finally, it has been found that mutated *LRRK2* has the ability to both increase alpha-synuclein levels and increase its recruitment into pathological inclusions (Volpicelli-Daley et al., 2016a).

1.2.4 Alpha-synuclein conformations in disease

Unfolded monomers are suggested as one source of alpha-synuclein toxicity, as the alpha-synuclein mutations A53T and E46K have been found to increase the monomeric conformation and decrease that tetrameric conformation (Dettmer et al., 2015a). Before becoming fibrils, alpha-synuclein oligomers, which have been found to be increased by a number of different factors including exposure to polyunsaturated fats (Sharon et al., 2003) and mildly acidic environments such as lysosomes and endosomes (Buell et al., 2014), undergo conformational change leading to the development of more stable and compact proteinase-K resistant oligomers which induce higher levels of oxidative stress (Cremades et al., 2012).

There is debate surrounding which conformation of alpha-synuclein is more toxic, oligomers or fibrils, with research providing support for each conformation. Soluble, lipid-dependent alpha-synuclein oligomers have been

observed in alpha-synuclein transgenic mice, and in PD and Dementia with Lewy bodies (DLB) patients brains (Sharon et al., 2003). Furthermore, increased formation of oligomers, and not fibrils, is observed with the A53T and A30P alpha-synuclein mutations (Conway et al., 2000). On the other hand, more recent research has suggested that it is fibrils, and not oligomers, which are the most toxic species of alpha-synuclein, leading to the greatest amount of motor impairment, dopaminergic cell loss and synaptic dysfunction following injection into the rat substantia nigra (Peelaerts et al., 2015).

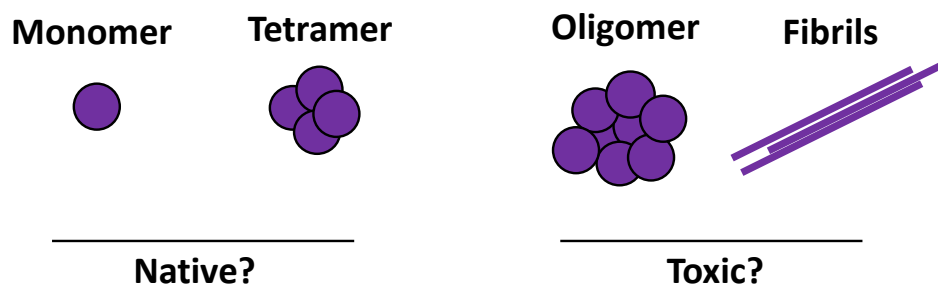


Figure 1.3: The main conformations of alpha-synuclein found in the brain. Adapted from (Wong and Krainc, 2017).

1.2.5 Lewy Bodies

Lewy bodies are intracellular inclusions which contain a variety of cellular proteins, the most highly abundant of which is alpha-synuclein. These amyloidogenic protein deposits also include Lewy neurites (Spillantini et al., 1997). Lewy bodies have been found to highly express alpha-synuclein which has been phosphorylated on serine-129, as well as many other post-translational modifications (Anderson et al., 2006). Extensive research has been conducted into the composition of Lewy bodies, and it has been found that there are more than 90 different molecules contained in these aggregates, including synphilin-1 (Wakabayashi and A., 2000), tau (Ishizawa et al., 2003), parkin (Schlossmacher et al., 2002), and LRRK2 (Zhu et al., 2006), to name but a few.

Lewy body pathology appears to be restricted to a subset of neurons. For example, in the midbrain and brainstem, Lewy bodies have the strongest

appearance in the dopaminergic neurons of the substantia nigra (Braak et al., 2004, Halliday et al., 1990). Assessment of the limbic system and cortex on the other hand, finds deposition of Lewy bodies to be mainly localised to the glutamatergic pyramidal neurons, particularly in layers 3 and 5 of the neocortex (Bernstein et al., 2011, Marui et al., 2003, Wakabayashi et al., 1995), and the cholinergic neurons in the basal forebrain (Dugger and Dickson, 2010). While Lewy bodies are mainly associated with alpha-synuclein, it is important to note that it has also been found that patients expressing mutations in other PD-associated genes, such as *LRRK2*, can also develop Lewy body pathology (Paisan-Ruiz et al., 2004, Zimprich et al., 2004, Trinh et al., 2014).

There has been a plethora of research conducted into the toxicity or otherwise of Lewy bodies and Lewy neurites, with no consensus reached. Conflicting results have been presented on the correlation of Lewy body burden with symptom severity (Beach et al., 2009, Harding et al., 2002, Mattila et al., 2000), and with neuronal dysfunction (Bergeron, 1996, Katsuse et al., 2003, Kramer and Schulz-Schaeffer, 2007). It has been proposed that this lack of agreement may be down to a number of factors, including the possibility that cells which are most vulnerable to Lewy body accumulation may die very rapidly, leaving only the Lewy body resistant neurons remaining, or there may be differences in composition across Lewy bodies, with some being more toxic than others. A final consideration is the possibility that it is the process itself during which the Lewy bodies are formed that is toxic rather than the Lewy bodies themselves, and that it is the smaller aggregations of alpha-synuclein that lead to cell death (Bras et al., 2020). It is important to note however, that Lewy pathology is also observed in a subset of neurologically healthy patients, known as incidental Lewy body disease, indicating that alpha-synuclein toxicity may not be wholly due to the presence of Lewy bodies (Frigerio et al., 2011).

1.2.6 Cell to cell spreading of alpha-synuclein in Parkinson's disease

The spreading of alpha-synuclein in PD has been proposed to be via a prion-like mechanism. It is suggested that toxic, self-propagating forms of alpha-synuclein have the ability to induce endogenous alpha-synuclein to be converted to a more pathological conformation in interconnected regions, leading to the propagation of the disease (Rey et al., 2016). Cell to cell spreading of alpha-synuclein has been observed in a number of different models and tissues, including in nigral neurons which were grafted into the brains of PD patients (Kordower et al., 2008, Li et al., 2008), through endocytosis to neighbouring neurons in cell culture and mouse models (Desplats et al., 2009), as well as through the recruitment of endogenous alpha-synuclein following exogenous alpha-synuclein expression in human cell lines and mouse neurons (Luk et al., 2009, Volpicelli-Daley et al., 2011).

The Braak staging model (Braak and Braak, 2000) posits that alpha-synuclein spreads both peripherally and centrally, impacting the enteric nervous system (ENS), the vagal nerve and the central nervous system (CNS). There has been much research into this model. For example, following injection of alpha-synuclein preformed fibrils (PFFs) into the duodenum and pyloric muscularis layer in the mouse, spreading of these fibrils was observed in the CNS, providing support for the enteric route hypothesis of alpha-synuclein spreading (Kim et al., 2019). Braak stages I and II propose that the earliest pathological changes associated with alpha-synuclein accumulation in PD begin in the olfactory bulb and medulla, progressively spreading as the disease progresses to stages III and IV, where it can be found in the midbrain and then the substantia nigra pars compacta, and finally stages V and VI, where it is found to be widespread throughout the brain (Braak et al., 2004).

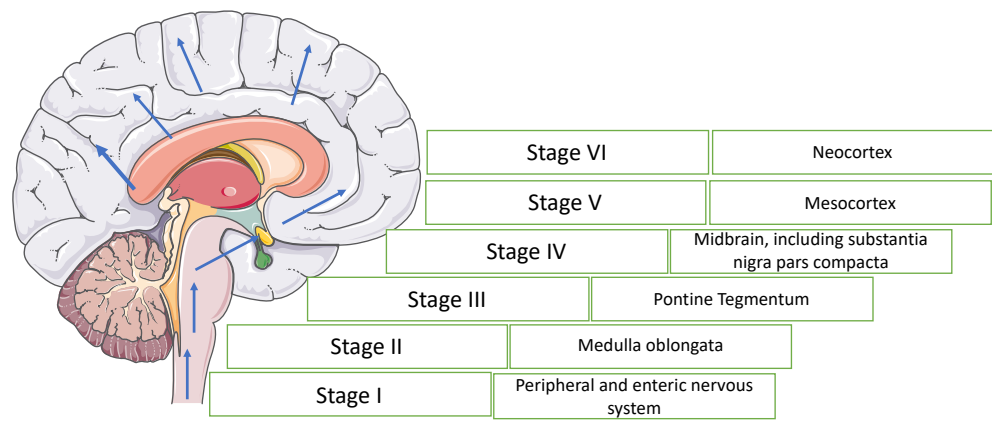


Figure 1.4: Illustration of the Braak staging model (Braak et al., 2004, Braak et al., 2003b), with arrows representing the proposed spreading pattern of alpha-synuclein as PD progresses.

The finding that the development of Lewy bodies in PD patients who have received grafts of embryonic mesencephalic neurons many years after grafting has led to the hypothesis that there are pathological forms of alpha-synuclein, and that it is these forms of alpha-synuclein that are responsible for the initiation and propagation of PD (Li et al., 2008, Kordower et al., 2008, Recasens et al., 2018). Furthermore, the presence of alpha-synuclein is not limited to the CNS. Lewy pathology has been observed in peripheral neuronal structures including the ENS (Kupsky et al., 1987, Wakabayashi et al., 1988) and sympathetic ganglia (Rajput and Rozdilsky, 1976).

1.2.7 Synucleinopathies

While the diseases associated with alpha-synuclein fall under the umbrella term of synucleinopathies, there are distinct variations between these diseases, in the way that they present clinically, as well as the cell types which are affected. For example, in PD, cell loss and the development of Lewy bodies is observed in multiple cell types including neuromelanin-positive catecholamine neurons in the substantia nigra and the locus coeruleus, the dorsal motor nucleus of the vagus neurons, as well as in norepinephrine and dopaminergic neurons of the enteric nervous system (Sulzer and Surmeier, 2013, Braak et al., 2003b). Other synucleinopathies include DLB and multiple systems atrophy (MSA) (Recasens et al., 2018). Furthermore, alpha-synuclein

deposits have also been found to be present in many other diseases, including AD, pure autonomic failure, and in rapid eye movement (REM) sleep behaviour disorder (Bras et al., 2020).

Comparison of the synucleinopathies PD, DLB, and MSA have identified some key differences. While PD and DLB show similar severity and distribution of alpha-synuclein, patients with DLB have significant cortical amyloid deposition (McCann et al., 2014). Contrastingly, patients with MSA show alpha-synuclein-positive glial cytoplasmic inclusions in oligodendrocytes. Some patients with MSA also show alpha-synuclein-positive neuronal cytoplasmic and nuclear, and glial nuclear inclusions (McCann et al., 2014). These patients develop autonomic failure, parkinsonism, and cerebellar ataxia, resulting from striatonigral and olivopontocerebellar degeneration (Fanciulli and Wenning, 2015).

PD with dementia presents with parkinsonism firstly, while DLB presents with dementia first, after which other symptoms will develop (Goldman et al., 2014). Alpha-synuclein Lewy body pathology and amyloid plaques are present in some but not all patients with PD with dementia and DLB, with a greater plaque expression reported in DLB as opposed to PD with dementia (Goldman et al., 2014). Alpha-synuclein toxicity has also been implicated in the development of lysosomal storage disorders, such as Gaucher's disease. Gaucher's disease is caused by mutations in Glucosylceramidase Beta 1 (GBA1) (Blanz and Saftig, 2016), and mutations in GBA1 are also risk factors for the development of PD (Sidransky et al., 2009). Other lysosomal storage disorders also show alpha-synuclein pathology, including Sanfilippo syndrome, GM2 gangliosidosis, and Niemann-Pick type C (Shachar et al., 2011). Finally, there are a group of genetic diseases known as neurodegeneration with brain iron accumulation. These diseases involve the accumulation of iron in the globus pallidus and substantia nigra, which leads to the development of symptoms of parkinsonism, spasticity and dystonia (Wong and Krainc, 2017). These patients can also present with Lewy bodies and neurites.

1.2.8 Alpha-synuclein interactions with cellular components in disease

Alpha-synuclein has been found to interact with a number of cellular components in disease, as illustrated in Figure 1.5. Studies in yeast have found that alpha-synuclein, both WT and mutated, leads to disruptions in endoplasmic reticulum to Golgi trafficking (Outeiro and Lindquist, 2003). Dopamine transporter (DAT) transport has also been found to be disrupted when alpha-synuclein is overexpressed (Oaks et al., 2013). It has been proposed that calcium buffering is disrupted by alpha-synuclein in the endoplasmic reticulum, as cytoplasmic calcium levels have been found to be raised in the presence of increased alpha-synuclein levels, with this cumulating in the activation of a calmodulin-calcium cascade, which has been found to be toxic (Caraveo et al., 2014, Luo et al., 2014). Furthermore, the transport of the autophagic transmembrane protein ATG9 from the endoplasmic reticulum to Golgi has been found to be disrupted when alpha-synuclein is overexpressed, with this alpha-synuclein overexpression also leading to decreased formation of omegasomes, which are precursors for autophagosome biogenesis (Winslow et al., 2010).

A53T and A30P alpha-synuclein have been found to bind to the lysosomal receptor lysosome-associated membrane protein 2 (LAMP2) much more tightly than WT alpha-synuclein, and this binding has been found to both prevent their own degradation, and also prevent chaperone-mediated autophagy and substrate cargo loading into lysosomes (Cuervo et al., 2004). It has been proposed that the selective dopaminergic vulnerability observed in PD is due in part to the fact that dopamine modified alpha-synuclein has also been observed to block chaperone-mediated autophagy (Martinez-Vicente et al., 2008). Research employing alpha-synuclein PFFs has found that when these PFFs are incubated with neurons, defective lysosomal fusion and autophagic cargo accumulation are observed, even though autophagosomes were observed to form normally (Tanik et al., 2013). It has been proposed that these alterations are due to defective autophagosome axonal transport (Volpicelli-Daley et al., 2014a). Through the use of induced pluripotent stem cells (iPSCs)

it has been found that, when compared with control iPSCs, those derived from PD patients with alpha-synuclein triplication showed decreased lysosomal activity for a number of enzymes as a result of defective endoplasmic reticulum to Golgi trafficking (Mazzulli et al., 2011, Mazzulli et al., 2016a, Wong and Krainc, 2016).

Through the use of alpha-synuclein fibrils it has been found that while the axonal transport of synaptophysin or mitochondria were not disrupted by the presence of these fibrils, the transport of autophagosomes was disrupted, leading to the conclusion that alpha-synuclein expression impairs some axonal transport, but not all (Volpicelli-Daley et al., 2014a). It has been suggested that alpha-synuclein impacts upon axonal transport through a number of different mechanisms including decreased expression of axonal transport proteins (Chu et al., 2012), decreased microtubule stability and kinesin-dependent cargo motility (Prots et al., 2013), interactions of alpha-synuclein with tau (Guo et al., 2013, Giasson et al., 2003), and disruption to actin turnover (Tilve et al., 2015).

Multiple pathways of cellular dysfunction have been reported in relation to alpha-synuclein toxicity. It is possible that different mutations of alpha-synuclein, as well as different synucleinopathies, develop different types of cellular dysfunction, and that these cellular dysfunctions change across the timeline of disease progression (Wong and Krainc, 2017). It is also important to consider the experimental nature of these studies, which leads to variations in the types of alpha-synuclein used, the models used to investigate alpha-synuclein toxicity, and the experimental outputs analysed.

SNCA, the gene encoding alpha-synuclein, is located on chromosome 4q22.1 and this gene is linked to an autosomal dominant inheritance pattern.

Furthermore it has been suggested that the nuclear localisation is regulated by the nuclear protein Tripartite motif-containing 28 (TRIM28), and that PD-associated mutations, when compared with wildtype (WT) alpha-synuclein, show increased nuclear localisation (Rousseaux et al., 2016, Kontopoulos et al., 2006, Fares et al., 2014).

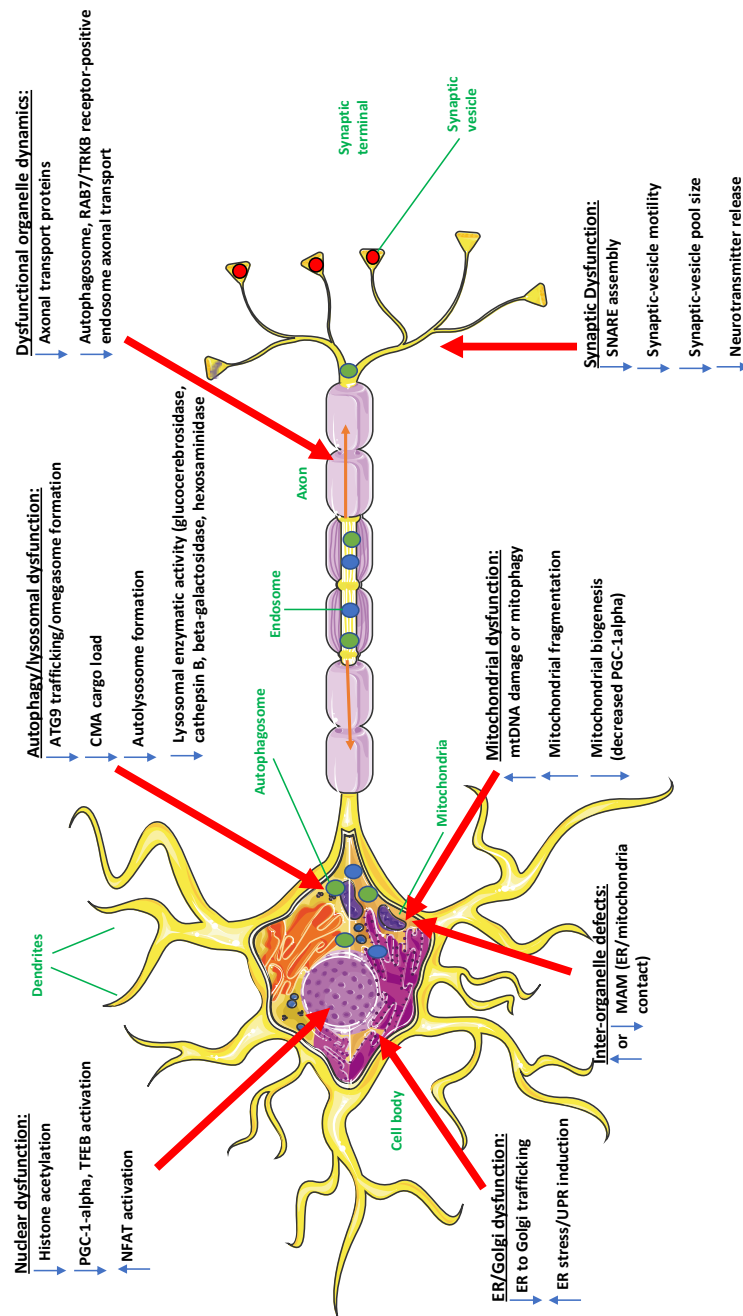


Figure 1.5: Proposed alpha-synuclein interactions with various cellular components in Parkinson's disease as described above. Image adapted from (Wong and Krainc, 2017). Transcription Factor EB (TFEB), Nuclear Factor of Activated T-cells (NFAT), Endoplasmic reticulum (ER), Unfolded Protein Response (UPR), mitochondria-associated-membranes (MAM), chaperone mediated autophagy (CMA).

1.2.9 Alpha-synuclein and synaptic vesicle trafficking in disease

There are differing schools of thought when it comes to whether or not alpha-synuclein's normal physiological role in the synapse is impacted in disease. Some studies argue that through the preferential binding of large alpha-synuclein oligomers to VAMP2 in PD, SNARE complex formation, release of dopamine, synaptic vesicle mobility are all disrupted, leading to decreased synaptic vesicle pool recycling, which ultimately cumulates in disruptions in neurotransmitter release (Scott and Roy, 2012, Choi et al., 2013, Wang et al., 2014, Nemani et al., 2010). However, other studies propose that SNARE assembly is in fact promoted by alpha-synuclein oligomers, and the majority of disease associated mutations of alpha-synuclein, with the exception of the A30P mutation, do not lead to the disruption of SNARE assembly or the clustering of synaptic vesicles (Diao et al., 2013, Burre et al., 2014, Burre et al., 2012).

Elevated levels of alpha-synuclein have been proposed to impact dopaminergic neurotransmission in a number of ways. Through a number of preclinical research studies, it has been found that in the presence of human alpha-synuclein, decreased dopaminergic terminals, defective dopaminergic release and altered synaptic vesicle function are all observed in the mouse brain (Masliah et al., 2000, Janezic et al., 2013). Furthermore, through the use of mice lacking alpha-synuclein it has been found that these mice have increased dopaminergic release from the nigrostriatal terminals, with the levels of cytosolic dopamine remaining comparable to mice without alpha-synuclein deletion (Abeliovich et al., 2000, Mosharov et al., 2009). Reduced dopamine reuptake and deficits in dopamine transporter function have also been linked to increased levels of alpha-synuclein expression in an adeno-associated viral (AAV) vector model of alpha-synucleinopathy in rats (Lundblad et al., 2012).

1.2.10 Alpha-synuclein and mitochondrial function in disease

The homeostasis of mitochondria is essential in a living cell, in order to allow for ATP-synthesis, lipid metabolism, the storage of calcium, and the survival of neurons (Nunnari and Suomalainen, 2012). It is therefore important to investigate the impact, if any, that alpha-synuclein has on mitochondria. Increased mitochondrial DNA damage and mitophagy has been observed in A53T alpha-synuclein mutant mice (Martin et al., 2006, Choubey et al., 2011, Chen et al., 2015b). Furthermore, Dynamin related protein 1 (DRP1) is essential for mitochondrial fission, which is essential to the creation of new mitochondria, as well as the removal of damaged mitochondria. Increased levels of alpha-synuclein, in both cell lines and mouse models has demonstrated that alpha-synuclein has the ability to promote mitochondrial fission independent of DRP1 (Kamp et al., 2010, Nakamura et al., 2011). However it is important that this promotion of mitochondrial fission is in balance with mitochondrial fusion, where mitochondria are joined together, as a disruption to this balance can lead to neuronal death.

It has been proposed that alpha-synuclein oligomers can promote increased calcium uptake leading to mitochondrial dysfunction, and this may be the reason why mice lacking alpha-synuclein have been found to be resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced dopaminergic neuronal degeneration (Dauer et al., 2002, Luth et al., 2014). Impaired mitochondrial protein transport has also been identified in response to alpha-synuclein with posttranslational modifications (Di Maio et al., 2016). It has also been proposed that alpha-synuclein toxicity may lead to decreased levels of the mitochondrial biogenesis factor PGC-1 α , which in turn leads to mitochondrial dysfunction. Decreased levels of PGC-1 α have been observed in mice expressing A30P alpha-synuclein, as well as in the brains of PD patients (Eschbach et al., 2015, Zheng et al., 2010).

Mitochondrial fragmentation has been reported when the impact of alpha-synuclein on the mitochondria-associated endoplasmic reticulum membrane

was investigated. However, there were differing conclusions regarding the impact of alpha-synuclein overexpression on mitochondria-associated-membranes (MAM). One study found that alpha-synuclein overexpression led to increased mitochondrial calcium uptake from the endoplasmic reticulum due to increased numbers of MAM sites (Cali et al., 2012). This finding however has been contradicted by research which has found that overexpression of wildtype, A30P or A53T alpha-synuclein led to decreased numbers of MAM contact sites alongside alpha-synuclein expression in MAM fragments (Guardia-Laguarta et al., 2014). More research is needed to untangle the impact of alpha-synuclein on mitochondrial fragmentation and inter-organelle contacts.

1.2.11 Why are dopaminergic neurons vulnerable?

The expression of dopaminergic neurons is usually assessed through immunohistochemistry for TH. Tyrosine hydroxylase catalyses the conversion of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), which undergoes a series of downstream enzymatic reactions in order to be processed into the neurotransmitter dopamine (Pickel et al., 1975) and is therefore a useful marker of the presence of dopaminergic cells (Alerte et al., 2008). Dopaminergic neurons are especially vulnerable to toxicity due to the propensity of dopamine to become oxidised within the cytoplasmic milieu (Hastings, 2009). Therefore it is proposed that any process which prevents the reuptake of dopamine or leads to the redistribution of dopamine from the vesicles leads to high levels of reactive oxygen species which affect many cellular processes including trafficking of organelles, autophagy and microtubule stability (Burke and O'Malley, 2013).

The size of dopaminergic neurons must also be taken into account, as well as their cellular components. It has been found that the mitochondria of dopaminergic neurons are approximately 40% smaller than those of other neurotransmitters, and move almost three times slower than mitochondria from non-dopaminergic neurons (Kim-Han et al., 2011, Liang et al., 2007). When the distance to the terminal fields in dopaminergic neurons are

considered in comparison to other neurotransmitters, they are generally far greater, which would mean that it would take a much longer time for the mitochondria to get to where they need to be when compared with other neurotransmitters (Matsuda et al., 2009).

It has been proposed that dopamine may enhance alpha-synuclein toxicity, as it has been found that the stabilization of alpha-synuclein protofibrils can be achieved through the use of dopamine adducts (Conway et al., 2001), which is proposed to lead to the inhibition of alpha-synuclein aggregation and the promotion of alpha-synuclein oligomer formation (Mazzulli et al., 2007, Mazzulli et al., 2006). Studies utilising midbrain dopaminergic neurons has found that dopaminergic neurons which are lacking alpha-synuclein are resistant to L-DOPA induced cell death, and that lower levels of cytosolic dopamine has neuroprotective effects (Mosharov et al., 2009).

1.2.12 Alpha-synuclein and neuroinflammation

Microglial activation, indicative of an inflammatory response, have been observed in multiple models of alpha-synucleinopathy (Lee et al., 2010, Theodore et al., 2008, Lastres-Becker et al., 2012, Gao et al., 2008, Harms et al., 2013). Multiple mechanisms have been reported for this increased inflammatory response observed following alpha-synuclein expression, including the activation of microglia through the agonist actions of extracellular alpha-synuclein released from neurons on TLR2 (Kim et al., 2013), and the potential chemoattractant properties which alpha-synuclein is suggested to exhibit, leading to the promotion of microglial migration (Wang et al., 2015). Furthermore, microglial activation and neuroinflammation have been observed in a number of models of alpha-synucleinopathy (Chung et al., 2009), as well as in patients with PD (Ouchi et al., 2005).

1.2.13 Interplay of alpha-synuclein with amyloid β and tau

As well as being implicated in PD, increased alpha-synuclein levels and the presence of Lewy body pathology is found in a subset of AD patients, with soluble alpha-synuclein in the brain found to correlate with the level of cognitive decline (Larson et al., 2012). Interestingly, the relationship between alpha-synuclein and amyloid β ($A\beta$) seems to be bidirectional. $A\beta$ has been found to enhance alpha-synuclein fibrilization both *in vitro* and *in vivo* (Masliah et al., 2001), and alpha-synuclein has been found to promote $A\beta$ 1-38 aggregation *in vitro* (Yoshimoto et al., 1995). Alpha-synuclein has also been found to regulate the fibrillization of tau and $A\beta$ (Bachhuber et al., 2015, Guo et al., 2013, Yoshimoto et al., 1995, Masliah et al., 2001, Giasson et al., 2003).

1.2.14 Alpha-synuclein and aging

Aging has been found to be the greatest risk factor for the development of multiple neurodegenerative diseases. Higher levels of mitochondrial DNA deletion is observed in substantia nigra neurons with age, leading to respiratory-chain deficiency which cumulates with mitochondrial dysfunction (Bender et al., 2006). Proteasome dysfunction has been found to increase with age in multiple cells types including neurons (Vilchez et al., 2014), with decreased efficiency of autophagy also observed with increasing age (Wong and Holzbaur, 2015). Interestingly, alpha-synuclein levels have also been found to increase with age in the human substantia nigra (Li et al., 2004, Chu and Kordower, 2007). Furthermore, oxidative stress has been found to increase with age (Finkel and Holbrook, 2000), which has been found to contribute to the nitration of alpha-synuclein (Giasson et al., 2000), which in turn promotes alpha-synucleins propensity to aggregate (Paxinou et al., 2001) and decreases its ability to bind lipids (Hodara et al., 2004).

1.2.15 Targeting alpha-synuclein therapeutically

1. Targeting alpha-synuclein synthesis

Preclinical research has investigated the impact of targeting alpha-synuclein synthesis through the use of small interfering ribonucleic acid (siRNA) which targets alpha-synuclein messenger ribonucleic acid (mRNA). In mice, decreased hippocampal and cortical alpha-synuclein levels were observed for one week following direct siRNA fusion (Lewis et al., 2008). Furthermore, decreased protein aggregation was observed in the substantia nigra of mice which expressed human alpha-synuclein following injection of siRNA-containing exosomes (Cooper et al., 2014). It has been proposed that as antisense oligonucleotide-mediated therapies are currently being tested in clinical trials for other neurodegenerative diseases, perhaps it would be beneficial to assess their use in synucleinopathies (Wong and Krainc, 2017).

2. Targeting alpha-synuclein protein degradation

Through enhancing alpha-synucleins lysosomal and/or autophagic degradation it may be possible to decrease the toxic effects of alpha-synuclein in neurodegenerative diseases. Research has been carried out in rats expressing alpha-synuclein, in which overexpression of TFEB, which is a lysosomal transcription factor, leads to decreased alpha-synuclein oligomeric expression and subsequent neurodegeneration (Decressac et al., 2013). Research in transgenic mice overexpressing alpha-synuclein has found that passive immunisation through antibodies against alpha-synuclein has two impacts. The first of these is the lysosomal clearance of alpha-synuclein, and the second is the targeting of alpha-synuclein to microglia, both of which cumulate in the reduction of alpha-synuclein induced neurodegeneration and behavioural deficits (Masliah et al., 2011, Bae et al., 2012). A body of research has been conducted into the role that Glucocerebrosidase (GCase) plays in alpha-synuclein degradation. GCase is a lysosomal glycoside hydrolase which cleaves the glycolipid glucosylceramide.

Multiple studies have reported its effectiveness in the degradation of alpha-synuclein including studies conducted in human neurons (Mazzulli et al., 2011), in mouse models of Gaucher's related synucleinopathy as well as several other models of alpha-synuclein toxicity (Sardi et al., 2013, Rocha et al., 2015, Rockenstein et al., 2016), and in human neurons derived from iPSCs (Mazzulli et al., 2016b, Aflaki et al., 2016). Patients with Gaucher's disease who have been receiving GCase enzyme replacement therapy for more than five years show decreased oligomeric alpha-synuclein levels when compared with patients not receiving enzyme replacement therapy (Pchelina et al., 2014). These findings suggest that through increasing alpha-synuclein protein degradation, some neuroprotective effects may be observed.

3. Targeting alpha-synuclein aggregation

Several clinical trials have investigated the use of small molecules to inhibit alpha-synuclein aggregation (glycerol phenylbutyrate; University of Colorado, Denver - ongoing) and nilotinib, a protein tyrosine kinase inhibitor (Georgetown University - ongoing) (Wong and Krainc, 2017). This research comes on the back of preclinical research which has found that mice with the A30P alpha-synuclein mutation show decreased soluble and membrane bound alpha-synuclein protofibrils in the spinal cord, accompanied by decreased motor dysfunction following passive immunisation using a protofibril-selective antibody (Lindstrom et al., 2014).

4. Targeting alpha-synuclein propagation

In alpha-synuclein mouse models, antibodies against C-terminal truncated alpha-synuclein are able to rescue motor and memory impairments, with *in vitro* research finding that these antibodies are effective at halting alpha-synuclein propagation (Games et al., 2014). Furthermore, research investigating the effects of monomeric alpha-synuclein antibodies in mice injected with alpha-synuclein PFFs have found that these antibodies prevent propagation and uptake of alpha-

synuclein, as well as rescuing dopaminergic cell loss and motor deficits (Tran et al., 2014).

5. Active immunisation against alpha-synuclein

Preclinical research has found reduced alpha-synuclein aggregates, improved motor and memory performance, and reduced dopaminergic degeneration and dopamine loss through the immunisation of a variety of alpha-synuclein transgenic mice with various forms of human alpha-synuclein antigen (Schneeberger et al., 2016, Masliah et al., 2005). Currently clinical trials are ongoing to assess the effectiveness of active immunisation against alpha-synuclein including the PD01A vaccine (AFFiRiS) which has shown safety and some clinical improvements (Schneeberger et al., 2016), as well as the assessment of passive immunisation against alpha-synuclein (Bergstrom et al., 2016) including PRX002, a monoclonal antibody (Prothena/Roche) which has shown safety and the ability to reduce alpha-synuclein levels in the blood. However not all studies have been effective, with BIIB054, a monoclonal antibody (Biogen) being terminated early as it did not meet its primary outcomes.

6. Targeting the immune system

In vitro research has investigated the impact of treatment with the hypertension drug Candesartan cilexetil, an angiotensin receptor blocker, on microglia treated with oligomeric alpha-synuclein and found a decreased proinflammatory response and inhibition of TLR2 expression (Daniele et al., 2015). Furthermore, research in alpha-synuclein toxicity models in rats found reduced dopaminergic neurodegeneration following treatment with either FK506, which is an immunosuppressant which acts through inhibition of calcineurin and modulation of mitochondrial function, or the janus kinase 1/2 (JAK1/2) inhibitor of the janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway AZD1480 (Van der Perren et al., 2015a, Qin et al., 2016). Finally, research in alpha-synuclein transgenic

mice has found reduced motor behaviour deficits and decreased microgliosis following treatment with hypoxoestride, which is a nuclear factor kappa B (NF- κ B) modulator, or lenalidomide, which is a NF- κ B signalling inhibitor, both of which are proposed to have anti-inflammatory effects (Valera et al., 2015, Kim et al., 2015).

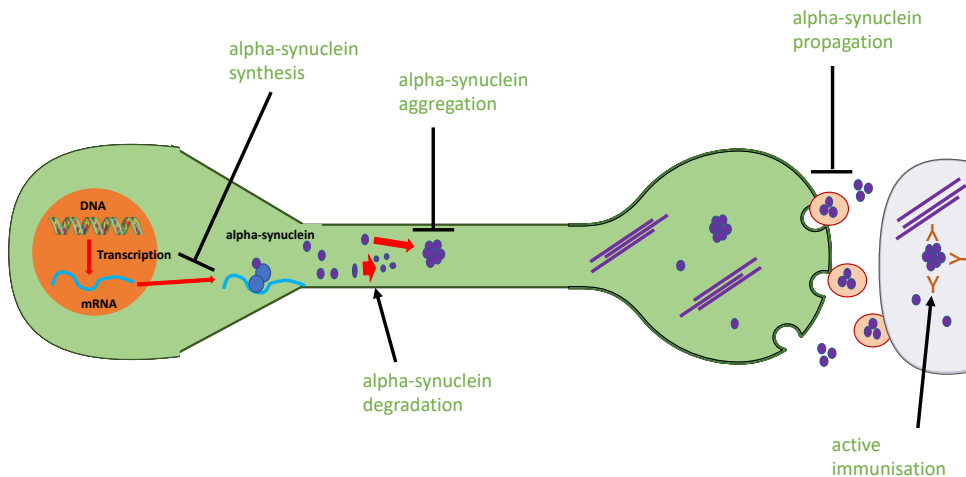


Figure 1.6: Proposed mechanisms for targeting alpha-synuclein as a potential treatment for PD. Adapted from (Wong and Krainc, 2017).

1.3 Animal models of Parkinson's disease

Animal models are essential tools in neuroscience research. They allow researchers to increase their knowledge surrounding the pathophysiology of various diseases, as well as testing novel compounds as therapeutic strategies for diseases which have no efficacious treatment and/or cure. Through animal models of PD the etiology, pathology and molecular mechanisms underlying PD have become better understood (Blesa et al., 2012).

Chemically induced models	Genetic models
MPTP	Transgenic models
6-OHDA	Adeno-associated viral (AAV) vector models
Rotenone	Preformed fibrils (PFF) models
LPS	AAV + PFF combination models
Paraquat	

Table 1.1: Examples of chemically induced and genetic models of PD.

1.3.1 Chemically induced models

Initial research into animal models of PD focused on the ablation of the dopaminergic nigrostriatal pathway through chemical means. This led to dramatic reductions of striatal dopamine, typical of that seen in late-stage PD, accompanied by motor deficits. Examples of these models include 6-hydroxydopamine (6-OHDA) and MPTP (Tieu, 2011). While these models have been useful in the development of dopamine replacement therapies, they do not account for the slow, progressive nature of PD, and therefore lack translatability to the human condition.

MPTP is lipophilic and readily crosses the blood-brain-barrier, where exposure to monoamine oxidase B leads to the conversion of MPTP to the toxic 1-methyl-4-phenylpyridium (MPP⁺). Through the DAT MPP⁺ enters dopaminergic neurons leading to a chain of actions ultimately culminating in significant increases in oxidative stress and resultant dopaminergic cell death (Schildknecht et al., 2015). While this is effective at leading to the degeneration of dopaminergic neurons in the substantia nigra and leading to the development of behavioural deficits, there are many disadvantages to this model being utilised as a model of PD. Not all rat strains are susceptible to MPP⁺ (Tieu, 2011, Meredith and Rademacher, 2011), with rats that are susceptible requiring direct injection into the substantia nigra (Di Matteo et al., 2006). Finally, MPTP does not lead to the generation of alpha-synuclein oligomers (Schober, 2004).

6-OHDA is a structural analogue of catecholamines, which has also been used to develop models of PD through damaging the nigrostriatal dopaminergic tract. Expression of 6-OHDA in the rat brain leads to oxidative stress through inhibition of complex-I activity in mitochondria and suppressing ATP production. It is hydrophilic and therefore must be delivered directly to the substantia nigra. It has been found to induce the classical motor symptoms associated with PD, accompanied by a loss of TH cells, with lower doses representative of the early phase of disease onset of prodromal symptoms such as anhedonia and memory deficits, in the absence of motor symptoms (Tadaiesky et al., 2008, Del Fabbro et al., 2019). As with MPTP, 6-OHDA does not lead to the development of Lewy bodies, one of the pathological hallmarks of PD, which is a major limitation of the use of this model when it comes to trying to untangle the pathological mechanisms underlying the development and progression of the disease.

As neuroinflammation has been implicated in the pathogenesis of PD, models have been developed to investigate the impact of inflammation on the midbrain and on motor behaviours. Lipopolysaccharide (LPS) has been used to develop an inflammatory model of Parkinsonism. LPS is a potent stimulator of microglia. Injection of LPS into the substantia nigra or striatum of rodents leads to neurodegeneration and motor deficits (Choi et al., 2009, O'Neill et al., 2020, Yssel et al., 2018). While LPS has been beneficial in elucidating the impact of neuroinflammation on motor behaviour and nigro-striatal dopaminergic integrity, it is a model with a very rapid onset of disease, and is therefore limited in its translatability to the human disease.

1.3.2 Transgenic models

Many transgenic models of PD have been created through promoters such as platelet derived growth factor B-chain (PDGF-B), mThy-1 mouse prion promoter (mPrP), and bacterial artificial chromosome (BAC-Tg) which drive the overexpression of either WT or mutated forms of alpha-synuclein in autosomal-dominant mouse models. Initial studies were limited in that alpha-synuclein expression was only observed in the brain areas expressing the transgene, with

these findings being most evident in transgenic models using dopamine neuron-specific TH promoters (Matsuoka et al., 2001, Rathke-Hartlieb et al., 2001, Richfield et al., 2002, Wakamatsu et al., 2007). These models express levels of alpha-synuclein comparable to human patients, however they lack the development of amyloidogenic alpha-synucleinopathy. Using a PITX3-IRES2-tTA construct, transgenic mice were generated with inducible expression of human mutant A53T alpha-synuclein (Lin et al., 2012). This study found that these animals develop some signs of neuropathology, including the presence of ubiquitin-alpha-synuclein, nigrostriatal dopaminergic neuron degeneration, as well as motor deficits. Interestingly alpha-synuclein expression was also observed outside of the expected regions, however this was attributed to the “leaky” expression of the transgene cassette.

Through the use of the TH promoter, signs of pathological accumulation of alpha-synuclein have been reported when the promoter drove the expression of a synthetic, aggregation prone, alpha-synuclein protein which lacked the last 20 amino acids (Tofaris et al., 2006). However this expression did not appear in neurons other than dopaminergic neurons. When considering these results it is important to take into account the fact that this mouse line expressed the transgene in a mouse mutant alpha-synuclein null background (Recasens et al., 2018). Other research utilising mouse strains which express exogenous alpha-synuclein drove the expression of C-terminally truncated alpha-synuclein and found no advanced alpha-synucleinopathy or spreading of alpha-synuclein past dopaminergic neurons (Wakamatsu et al., 2008, Daher et al., 2009). These models are limited in their effectiveness as they have been found to lack the ability to lead to the normal spatiotemporal expression in PD, with variability also observed in the stability and templating ability of alpha-synuclein (Visanji et al., 2016). Advanced alpha-synucleinopathy has been observed in transgenic mouse lines in which alpha-synuclein was expressed under the control of pan-neuronal promoters PPR, Thy1, with this alpha-synucleinopathy observed in the brainstem and spinal cord. These mice develop a motor phenotype as they age which becomes lethal (Giasson et al., 2002, Lee et al., 2002, Neumann et al., 2002). It has been found that this alpha-synucleinopathy remains largely in

the brainstem throughout the lifespan of these mice, perhaps suggesting that this is a model of Braak stage II and does not progress further.

1.3.3 Use of alpha-synuclein derived from humans to generate animal models of alpha-synucleinopathy

Assessment of the ability of human brain samples derived from patients with DLB to induce pathology in animal models found that alpha-synuclein pathology could be induced following the inoculation of these human brain samples into the mouse brain (Masuda-Suzukake et al., 2013). More specifically, at 15 months post-injection 50% of the female C57BL/6J mice that received intra-nigral injection of these human brain samples exhibited expression of phosphorylated alpha-synuclein pathology in many regions of the brain, including the substantia nigra, striatum, hippocampus, amygdala, hypothalamus, motor cortex, somatosensory area, piriform cortex, and superior colliculus. However, when compared with the animals injected with recombinant insoluble human or mouse alpha-synuclein fibrils, it was found that 100% of these animals also expressed phosphorylated alpha-synuclein pathology (Masuda-Suzukake et al., 2013).

Brain homogenates from MSA patients were injected into the brains of TgM83^{+/-} mice. These mice express low levels of A53T alpha-synuclein and they do not develop signs of spontaneous neurological illness (Watts et al., 2013). Following injection of brain homogenates from MSA patients in the right parietal lobe these animals displayed ataxia and circling behaviour at approximately 120 days post-injection. Neuropathologically, these mice exhibited astrocytic gliosis, increased numbers of activated microglia, increased levels of detergent-insoluble phosphorylated alpha-synuclein which was sensitive to proteinase-K, and widespread expression of pathological alpha-synuclein (Watts et al., 2013).

The impact of the injection of alpha-synuclein-expressing Lewy body fragments from patients with PD into the substantia nigra of WT mice has also been assessed. Four months following this injection mice developed nigrostriatal

degeneration and astrogliosis, with this continuing to 17 months post-injection (Recasens et al., 2014). Furthermore, it was found that this expression of alpha-synuclein led to increased endogenous intracellular alpha-synuclein levels, the conformational change of soluble endogenous alpha-synuclein into aggregated proteinase-K resistant forms, and increased alpha-synuclein phosphorylated at serine129 in interconnected regions such as the striatum. In order to control for the impact of the delivery of these samples from humans, similar samples lacking alpha-synuclein were administered to control animals, with no pathology presenting following this injection (Recasens et al., 2014).

Research in non-human primates has also found that injection of human derived Lewy-body enriched fragments into the substantia nigra or striatum leads to the development of striatal dopaminergic terminal loss and the expression of alpha-synuclein pathology (Recasens et al., 2014). Interestingly, while the animals injected in the striatum exhibited widespread alpha-synuclein pathology and expression of phosphorylated alpha-synuclein in several brain regions, injection into the substantia nigra led to a more local alpha-synuclein pathology at the site of injection.

Increasing awareness is developing surrounding the role of the gastrointestinal system in PD. This is due to the growing evidence that PD patients exhibit gastrointestinal alterations and dysbiosis. Investigations into the impact of the administration of faecal microbiota from PD patients by oral gavage into Thy1 alpha-synuclein-overexpressing ASO mice in germ-free conditions found that these mice developed increased motor dysfunction when compared with mice administered healthy control microbiota (Sampson et al., 2016). Interestingly, when these faecal microbiota from PD patients were administered to WT mice, no behavioural deficits were observed.

1.3.4 AAV models of disease

Viral vectors can be used as gene delivery systems as viruses have been found to have the ability to deliver exogenous nucleic acids to cells (Robbins and Ghivizzani, 1998). In order for vectors to be effective at gene delivery in the adult central nervous system (CNS) they must have the ability to transduce non-dividing cells, demonstrate low pathogenicity, and allow for long term gene expression. Much research has been conducted into the use of viral vectors as tools to induce expression of proteins of interest, in this case alpha-synuclein, in the rodent brain. Initial research focused on the use of either recombinant AAV vector or lentiviral vector intra-nigral injection into the rat brain (Kirik et al., 2002, Klein et al., 2002, Lo Bianco et al., 2002). While both vector types led to the long-term expression of alpha-synuclein, AAV vectors were found to be more efficient, leading to the transduction of approximately 95% of nigral dopaminergic neurons, and had a more precise targeting of neuronal cells (Ulusoy et al., 2010a, Decressac et al., 2012b, Low and Aebischer, 2012). Furthermore, the AAV has been found to have a much higher tropism for substantia nigra neurons, making it more suitable for the development of animal models of PD (Kirik et al., 2002).

The use of AAV vector models in preclinical PD research has many advantages. They have been found to induce PD like pathology in the form of cytoplasmic inclusions containing alpha-synuclein, alpha-synuclein-positive dystrophic neurites, as well as post-translationally modified forms of alpha-synuclein such as nitrated and Ser129-phosphorylated alpha-synuclein (Yamada et al., 2004, Koprach et al., 2010, Ulusoy et al., 2010b, Daher et al., 2015). Ser129-phosphorylated alpha-synuclein has been suggested to affect alpha-synuclein's solubility, membrane binding properties, and subcellular distribution (Rocha et al., 2018). Alpha-synuclein phosphorylation at Ser129 is mediated by protein kinases such as Polo-like kinase 2 (PLK2), which acts only on alpha-synuclein, not beta or gamma synuclein, and can therefore be used as a marker of alpha-synuclein aggregation.

AAV vector models have also been found to induce the accumulation of high molecular weight alpha-synuclein species which is detectable through both immunohistochemistry and biochemistry (Azeredo da Silveira et al., 2009, Koprach et al., 2010, Taschenberger et al., 2012, Oliveras-Salva et al., 2013, Ulusoy et al., 2017). Synaptic dysfunction has also been observed in AAV models inducing the expression of alpha-synuclein (Lundblad et al., 2012, Tozzi et al., 2016), as well as reductions in striatal dopamine (Ulusoy et al., 2010b, Decressac et al., 2012b, Oliveras-Salva et al., 2013, Bourdenx et al., 2015). Interestingly, AAVs have also been used to assess the proposed alpha-synuclein propagation from neuron to neuron. Through direct injection of AAVs carrying human alpha-synuclein DNA into the vagus nerve of rats this study found that alpha-synuclein expression could be observed in the pons, the midbrain and the forebrain (Ulusoy et al., 2013). Studies have built upon this finding and have demonstrated similar effects in mice (Helwig et al., 2016). Tables 1.2 and 1.3 illustrate studies which have utilised AAV vectors to generate preclinical models of PD using either WT or A53T human alpha-synuclein.

AAV WT human alpha-synuclein						
Author	AAV serotype and construct	Injection Volume and Titre	Control	Strain and Sex	TH Cell loss in alpha-synuclein group	Behavioural Deficits in alpha-synuclein group
Kirik et al., 2002	rAAV serotype not reported, CBA promoter with CMV enhancer elements	2µl; 8.2 x 10 ¹¹ inducing units/ml	rAAV GFP	Sprague Dawley; Not reported	Yes, at 3 and 8 weeks post-AAV	Yes, in Stepping Test at 8 weeks post-AAV and only after TH enzyme inhibitor treatment
Yamada et al., 2004	rAAV, serotype not reported, CMV promoter	3 µl; 4.2 x 10 ¹¹ genomes/ml	rAAV GFP serotype not reported	Sprague Dawley; Male	Yes, at 13 weeks post-AAV	No differences in Apomorphine Rotation Test at 12 weeks post-AAV delivery
Gorbatyuk et al., 2008	rAAV2/5, CBA promoter with CMV enhancer elements	1.5µl; 0.9 x 10 ¹³ viral genomes/ml	rAAV2/5 GFP	Sprague Dawley; Not reported	Yes, at 8 and 26 weeks post-AAV	Not reported
McFarland et al., 2009a	rAAV2/8, CBA promoter, WPRE element	2µl; 1.4 x 10 ¹⁰ genome copies/ml	rAAV2/8 empty*	Sprague Dawley; Not reported	Yes, at 6 weeks post-AAV	Not reported
Gorbatyuk et al., 2010	AAV5, CAG promoter	1.5µl; ~0.5–10x 10 ⁹ vector genomes	rAAV GFP	Sprague Dawley; Not reported	Yes at 8 weeks post-AAV	No effects in Amphetamine Induced Rotation Test from 4 -26 weeks post-AAV
Sanchez-Guajardo et al., 2010	rAAV2/5, CBA promoter	2µl; 6.7x10 ¹³ genome copies/ml; 6.2x10 ¹³ genome copies/ml	rAAV2/5 GFP	Sprague Dawley; Female	Yes at 4, 12 and 14 weeks post-AAV	Not reported
Decressac et al., 2011	AAV2/6, CAG promoter	3-4.5µl; 3.7 x 10 ¹³ genome copies/ml	AAV2/6 GFP	Sprague Dawley; Female	Yes, at 8 weeks post-AAV	Yes, in the Amphetamine Induced Rotation Test at 8 weeks post-AAV
Decressac et al., 2012a	AAV2/6, synapsin1 promoter, WPRE element; or AAV2/6, CBA promoter (no WPRE element)	3µl; 3.1 x 10 ⁸ genome copies/3µl	AAV2/6 GFP	Sprague Dawley; Female	Yes, at 3 and 8 weeks post-AAV in the WPRE group	Yes, in the Stepping Test at 3 weeks post-AAV; in the Stepping, Cylinder and Amphetamine Rotation Test at 8 weeks post-AAV in the WPRE group
Gaugler et al., 2012	rAAV2/6, WPRE element	Virus dose of 2.8 x 10 ⁷ transducing units	rAAV2/6 GFP	Sprague Dawley; Female	Yes, at 4 months post-AAV	Yes, in the Cylinder Test and Apomorphine Totation Test at 3 months post-AAV
Taschenberger et al., 2012	AAV2, human synapsin1 promoter, WPRE element, bpA	2µl; 1.2 x 10 ⁸ transducing units	AAV2 GFP	Wistar; Female	Yes at 2, 4, 8 and 14 weeks post-AAV	Not reported
Gombash et al., 2013	rAAV2/5, CAG promoter	4 µl (2 injections); 1.0 x 10 ¹³ ; or 4 µl (2 injections); 1.0 x 10 ¹⁴ viral genomes/ml	rAAV2/5 GFP	Sprague Dawley; Male	Yes, at 4 and 8 weeks for 1.0 x 10 ¹⁴ group, at 8 weeks for 1.0 x 10 ¹³ group	Yes, difference in 1.0 x 10 ¹³ group in the Cylinder and Adjusting Steps Test at 8 weeks post-AAV. No differences at 4 weeks in either titre group

Table 1.2: Studies using AAV expression of WT alpha-synuclein in rats. bpA: bovine growth hormone and polyadenylation site; AAV: adeno-associated virus, WT: wildtype, CBA: chicken beta actin, CMV: cytomegalovirus promoter; GFP: Green Fluorescent Protein; CAG: chicken beta-actin cytomegalovirus hybrid, TH: tyrosine hydroxylase, WPRE: Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element. * injected into opposite hemisphere.

AAV human A53T alpha-synuclein						
Author	AAV serotype and construct	Injection Volume and Titre	Control	Strain and Sex	TH Cell loss in alpha-synuclein group	Behavioural Deficits in alpha-synuclein group
Kirik et al., 2002	rAAV serotype not reported, CBA promoter with CMV enhancer elements	2µl; 1.4 x 10 ¹² inducing units/ml	rAAV GFP serotype not reported	Sprague Dawley; Not reported	Yes, at 3 and 8 weeks post-AAV	Yes, in Stepping Test at 8 weeks post-AAV and only after TH enzyme inhibitor treatment
Chung et al., 2009	AAV2, synapsin promoter	2 x 2µl injections; 1.8 x 10 ¹² genome copies/ml	AAV2 GFP	Sprague Dawley; Female	Yes, at 17 weeks post-AAV and at 24 weeks post-AAV	Not reported
Koprich et al., 2010	AAV1/2, CAG promoter with WPRE element, bpA	2µl; 5.1 x 10 ¹² genomic particles/ml	AAV1/2 GFP and AAV1/2 Empty	Sprague Dawley; Female	Yes, at 3 weeks post AAV, in both A53T and GFP when compared to Empty	Not reported
Koprich et al., 2011	AAV1/2, CAG promoter with WPRE element, bpA	2µl; 1.7 x 10 ¹² genomic particles/ml; 2µl; 5.1 x 10 ¹¹ genomic particles/ml	AAV1/2 GFP and AAV1/2 Empty	Sprague Dawley; Female	Yes, only in the 1.7 x 10 ¹² titre group at 6 weeks post-AAV	Yes, in the Cylinder Test at 3 weeks post-AAV in both titre groups; at 6 weeks in the 1.7 x 10 ¹² group only.
Gaugler et al., 2012	rAAV2/6, WPRE element	Virus dose of 2.8 x 10 ⁷ transducing units	rAAV2/6 GFP	Sprague Dawley; Female	Yes, at 4 months post-AAV	Yes, in the Cylinder Test and Apomorphine Rotation Test at 3 months post-AAV
Bourdenx et al., 2015	AAV2/9, CMV promoter	2 µl; 7.0 x 10 ¹² viral genomes/ml	'healthy' rats	Sprague Dawley; Not reported	Yes, starting at 1 week post-AAV delivery up to 16 weeks post-AAV	Yes, in the Stepping Test at 4 weeks post-AAV delivery, and in Spontaneous Activity at 8 weeks post-AAV
Rocha et al., 2015	AAV2/5	2 x 2µl injections; 2.0 x 10 ¹² genome copies/ml (GFP alone, or GFP + A53T)	AAV2/5 GFP	Sprague Dawley; Female	Yes, at 24 weeks post-AAV in GFP + A53T group	Not reported

Table 1.3: Studies using AAV expression of A53T alpha-synuclein in rats. bpA: bovine growth hormone and polyadenylation site; AAV: adeno-associated virus, CBA: chicken beta actin, CMV: cytomegalovirus promoter; GFP: Green Fluorescent Protein; CAG: chicken beta-actin cytomegalovirus hybrid, TH: tyrosine hydroxylase, WPRE: Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element.

1.3.4.1 Generation of AAV vectors

The AAV virus is a small non-enveloped single strand DNA (ssDNA) virus, lacking in pathogenicity, which can infect both dividing and quiescent cells. It comes from the dependoparvovirus genus of the parvovirus family. It has a number of advantages for use as a viral vector including its lack of pathogenicity, low efficiency for transducing antigen presenting cells, and its ability to transduce both dividing and non-dividing cells (Nayak and Herzog, 2010). The AAV contains two genes, *rep* and *cap*, which are flanked by two inverted terminal

repeats (ITRs) (Flotte, 2004). Proteins involved in viral replication are coded for in the *rep* region, while the *cap* region codes for capsid proteins (Schultz and Chamberlain, 2008). Cis-acting sequences, which are necessary for the packaging, replication and integration of the virus into the host genome are contained in the ITRs (Flotte, 2004). Through the deletion of portions of the viral *rep* and *cap* genes, and replacing them with the transgene of interest, the recombinant AAV is designed (Flotte, 2004). The immune response to the recombinant virus is greatly reduced, due to the minimal expression of viral proteins (Kirik et al., 2002).

Adeno-associated viral vectors used in preclinical research are constructed using a transfer plasmid carrying AAV ITRs, with a promoter downstream to control gene expression (Decressac et al., 2012b). The expression of the transgene is driven by multiple factors including the serotype, titre and promoter used. There are many different serotypes of AAV. A serotype is a variation of the virus that is not recognised by the same neutralizing antibodies (Weinberg et al., 2013). There are twelve known serotypes, differentiated mainly by the surface-exposed regions of the capsid, which results in variable host-vector reactions (Lisowski et al., 2015). In order for optimal cell internalization to occur, the AAV binds to a primary receptor, while also interacting with various co-receptors, with the tropism of the AAV determined by its affinity for the cell surface receptor, as well as post-internalization molecular pathways (Lisowski et al., 2015). There have been many advances in the design of recombinant AAV vector designs, including the generation of hybrid serotypes, which involves cross-packaging of one serotype with the capsid genes of another serotype, allowing for enhanced cell transduction (Lisowski et al., 2015). In hybrid serotypes one serotype capsid is swapped for another in order to enhance efficiency. As AAV2 is the best-characterised serotype it is found in most hybrid models as a core component, such as AAV1/2 and AAV2/5 (Carta et al., 2020).

AAV Serotype	Known Receptor	CNS Cell Tropism
AAV1	N-linked sialic acid	Neurons Oligodendrocytes Astrocytes
AAV2	Heparin Sulfate Proteoglycan	Neurons
AAV5	N-linked sialic acid	Neurons (high efficacy) Astrocytes
AAV6	Epidermal growth factor receptor Heparin	Neurons
AAV8	37/67 kd laminin receptor	Neurons (high efficacy) Astrocytes
AAV9	37/67 kd laminin receptor	Hippocampal neurons (high efficacy)

Table 1.4: Adeno-associated viral vector serotypes, including known receptors and CNS cell tropism. Data compiled from (Weinberg et al., 2013, Schultz and Chamberlain, 2008, Lisowski et al., 2015, Aschauer et al., 2013).

Early research utilising AAV vectors were limited due to the small AAV genome size of below five kilobases, which restricted expression cassette design as cell specific promoters tended to occupy much of the viral genome (Shevtsova et al., 2005). Vector design was greatly improved by the use of short promoters such as human synapsin-1, which allows for neuron specific vector targeting, and cytomegalovirus (CMV). Other improvements include the use of the chicken-beta-actin/cytomegalovirus hybrid promoter (CAG), and enhancer elements such as woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) (Shevtsova et al., 2005). Generally, in order to generate recombinant AAV vectors human embryonic kidney (HEK293) cells are transfected with plasmids, a transfer plasmid containing the gene of interest inserted between the ITRs, a plasmid containing the AAV *rep* and *cap* genes, and a plasmid encoding the factors that facilitate transfection. The virus is then purified, concentrated, and titred by real-time polymerase chain reaction (rtPCR) (Volpicelli-Daley et al., 2016a).

Promoters such as chicken beta actin (CBA) and CMV are used to control gene expression, both of which are strong promoters and drive expression of the gene. Additional elements are commonly added to the vector construct, including WPRE, bovine growth hormone and polyadenylation sequence, in order to encourage the transcription of the viral nuclear material carried by the

AAV vector. When the AAV vectors are injected into the brain they enter the cell through receptor-mediated endocytosis. They are then transported to the nucleus via a clathrin-coated vesicle, where they release the genetic information. This is then copied from DNA into mRNA via transcription, and through translation the associated protein is synthesised from the information in the mRNA, leading to the expression of the protein in the animal.

1.3.5 Preformed fibrils models of disease

In recent years, the use of PFFs to initiate the seeding and propagation of alpha-synuclein have been investigated in preclinical models of PD. Varying extents of toxicity are induced by both oligomeric and fibrillar alpha-synuclein, however fibrils have been suggested to be the most successful conformation of alpha-synuclein when it comes to the propagation of the pathology (Gomez-Benito et al., 2020). Preformed fibrils are generated through recombinant alpha-synuclein monomers, which are aggregated into fibrils. Much research has been conducted into the use of alpha-synuclein PFFs for the generation of preclinical models of PD. They have been found to induce aggregation, ubiquitination and hyperphosphorylation in the brain (Gomez-Benito et al., 2020). In primary hippocampal neurons derived from WT C57BL6 mice, recombinant alpha-synuclein PFFs show the ability to internalize and induce the recruitment of endogenous alpha-synuclein into insoluble pathological inclusions (Volpicelli-Daley et al., 2011). Endogenous alpha-synuclein is crucial for the development of pathology following the injection of some form of pathological alpha-synuclein. For example, injection of Lewy-body enriched fragments isolated from PD patients did not lead to the development of pathology or nigrostriatal degeneration in transgenic mice which did not express alpha-synuclein (Recasens et al., 2014).

Some researchers have investigated the impact of the delivery of AAV alpha-synuclein in combination with PFF alpha-synuclein. Aggregates formed following PFF alpha-synuclein administration incorporate endogenous alpha-synuclein (Thakur et al., 2017). Models which utilise the delivery of two forms

of alpha-synuclein, for example inducing expression following AAV injection in combination with the injection of PFFs, attempt to capitalise on this seeding propensity and generate models which develop faster than that seen with one form of alpha-synuclein expression, with greater pathology and motor deficits.

1.3.6 Caveats when interpreting results from preclinical studies utilising alpha-synuclein

There is great variability in the results obtained in preclinical research into synucleinopathies and there are many reasons for this. Studies can vary in relation to the genotype of the host, for example some research will employ WT animals, while others work with transgenic animals. There have been many administration routes utilised when alpha-synuclein is being administered including intra-nigral, intra-striatal, intra-muscular etc. Furthermore, it is important to consider the preparation of the alpha-synuclein and the controls used. In studies in which alpha-synuclein was obtained from human brain samples there are a variety of ways in which it could be produced, including total brain homogenates or Lewy body-bearing fractions.

Before injection of alpha-synuclein, it may have been stored under different conditions or have undergone sonication. For example, when reviewing the research conducted with alpha-synuclein fibrils or oligomers, it must be considered that if there were variations in the preparation techniques employed for the fibrils, then the fibrils or oligomers used across studies could vary dramatically in relation to their seeding, propagation, and toxicity-inducing capabilities (Guo et al., 2013, Bousset et al., 2013). During the administration of alpha-synuclein factors such as the volume of injection and the speed at which the injection was performed may vary across studies. Studies also vary when it comes to the timepoint post-injection at which the behavioural and post-mortem observations were made (Recasens et al., 2018). Interpreting results from studies utilising AAV vectors and comparing their results to other studies is difficult for a number of reasons including the titre of the AAV used, the AAV capsid serotype used, the control used, the length of time after

injection before the behavioural and post-mortem measures were made and the animal which was injected with the AAV.

When considering transgenic mouse models of alpha-synucleinopathy it is important to consider the factors which may lead to the limitation of the spreading of alpha-synuclein pathology. It is possible that there are compensatory mechanisms at play which limit the spreading of alpha-synuclein in the prion-like manner proposed in humans, and observed in some animal models in which the expression of alpha-synuclein was induced in adult animals through injection (Recasens et al., 2018). Furthermore, results from AAV studies vary in relation to the amount of dopaminergic neurodegeneration observed, which can range from 10% to 80% (Klein et al., 2002, Eslamboli et al., 2007, St Martin et al., 2007, Van der Perren et al., 2011, Ulusoy et al., 2012, Oliveras-Salva et al., 2013). Finally, while research utilising human iPSCs have the benefit of being able to utilise endogenous levels of alpha-synuclein, however these are cell models and lack the connectivity observed in the brain (Wong and Krainc, 2017).

1.4 Objectives of the thesis

The aim of this project is to assess the impact of over-expression of human alpha-synuclein, either through the use of AAV vectors or PFFs, in the rat substantia nigra, and the utility of this in the potential generation of a rat model of synucleinopathy. Specifically, the main objectives were:

- (1) The establishment and characterisation of a model of synucleinopathy in the rat induced by unilateral administration of AAV5-CBA-alpha-synuclein (human WT). Here we aim to assess the impact of delivery of AAV5-CBA-alpha-synuclein (human WT) to the substantia nigra on alpha-synuclein immunofluorescence and motor function at 12 and 16 weeks post-AAV delivery.
- (2) The establishment and characterisation of a model of synucleinopathy in the rat induced by unilateral administration of AAV1/2-CMV-alpha-synuclein (human A53T). Here we aim to assess the impact of delivery of AAV1/2-CMV-alpha-synuclein (human A53T) to the rat substantia nigra on alpha-synuclein immunofluorescence, TH immunoreactivity and associated changes in motor function at 3.5, 6.5 and 13 weeks post-AAV delivery. Furthermore, both male and female rats are assessed, in order to determine potential sex-based differences which may arise.
- (3) The establishment and characterisation of a model of synucleinopathy in the rat induced by unilateral administration of AAV1/2-CMV-alpha-synuclein (human A53T) in combination with A53T human PFFs. Here we aim to assess the impact of delivery of AAV1/2-CMV-alpha-synuclein (human A53T) in combination with A53T human PFFs to the rat substantia nigra on alpha-synuclein immunofluorescence, TH immunoreactivity and associated changes in motor function six weeks post-AAV plus PFF delivery. Furthermore, both male and female rats are assessed, in order to determine potential sex-based differences which may arise.

2. Materials and Methods

2.1 Materials

Laboratory Equipment

Material	Supplier
Microscope Digital Camera (Olympus DP72)	OLYMPUS
Perfusion Pump	Gilson ®
Fisherbrand™ GP1100 General Purpose Peristaltic Pump System	Fisher Scientific
Olympus BX51 Light Microscope	Olympus
Olympus Ix81 fluorescence microscope	OLYMPUS
Andor iXon+ ultra-sensitive EMCCD camera	Andor
Cryostat	Lecia
Gyro Rocker	Stuart, Ireland
pH meter	Mettler Toledo

Laboratory Chemicals and Reagents

Material	Supplier
Absolute Ethanol (EtOH)	HAZMAT Trinity College Dublin (TCD)
Chromium (III) potassium sulfate (KCr(SO ₄) ₂)	Fisher Scientific
Deionised Water	TCD
Ethylene Glycol	Sigma-Aldrich
Gelatin	Sigma-Aldrich
Hydrochloric Acid (HCl)	Sigma-Aldrich
Isopentane (2-methylbutane)	VWR
Medical Oxygen (O ₂)	BOC
Methanol	Sigma-Aldrich
Paraformaldehyde (PFA)	Sigma-Aldrich
Sodium Chloride (NaCl)	Sigma-Aldrich
Sodium Hydroxide (NaOH)	Sigma-Aldrich
Sodium dihydrogen phosphate (NaH ₂ PO ₄)	Sigma-Aldrich
Sodium phosphate dibasic (Na ₂ HPO ₄)	Sigma-Aldrich
Sucrose	Sigma-Aldrich
OCT compound	VWR International

Laboratory Plastics and Consumables

Material	Supplier
Coverslips (glass, 13mm)	VWR International
7ml bijoux tubes	Fisher Scientific
Falcon Tubes (15ml and 50ml)	Sarstedt
Eppendorf tubes (1.5ml and 2ml)	Sarstedt
Glass coverslips (22mmx60mm)	Fisher Scientific
Laboratory roll	Fisher Scientific
Microscope Slides	VWR
Microtome blades (c35 type)	VWR
Pasteur pipettes (3.5ml)	Sarstedt
Pipette tips	Sarstedt
Pipette tips filtered	Medical Supply Company
Petri dishes (92mm)	Sarstedt
24 well plates	Fisher Scientific
6 well plates	Fisher Scientific
Scalpels, disposable, sterile (Swann-Morton)	Fisher Scientific

Adeno-associated Viral Vectors and Pre-formed Fibrils

Material	Supplier
AAV5-CBA-eGFP	Viral Vector Core, University of Iowa, USA
AAV5-CBA-human wildtype alpha-synuclein	Viral Vector Core, University of Iowa, USA
AAV2/5-CBA-empty	Viral Vector Core, University of Iowa, USA
AAV1/2-CMV-A53T alpha-synuclein (GD1001-RV)	Vigene Biosciences, Maryland, USA
AAV1/2-CMV-Null/Empty (GD1004-RV)	Vigene Biosciences, Maryland, USA
AAV1/2-CAG-miR scrambled control alpha synuclein co-expressing eGFP (GD1009-RV-C)	Vigene Biosciences, Maryland, USA
WT alpha-synuclein (SPR-322) Type1 PFFs; human	Stressmarq Biosciences, Victoria, BC, Canada
A53T alpha-synuclein (SPR-326) Type1 PFFs; human	Stressmarq Biosciences, Victoria, BC, Canada

Animals

Material	Supplier
Male Wistar Han Rats	Envigo, UK
Male Wistar Han Rats	Comparative Medicine TCD
Female Wistar Han Rats	Envigo, UK
Female Wistar Han Rats	Comparative Medicine TCD
Standard Rat Chow	Comparative Medicine TCD
Cage Enrichment	Comparative Medicine TCD

Stereotaxic Surgery

Material	Supplier
Isoflurin (Isoflurane)	Comparative Medicine TCD
MSS-3 Isoflurane Vaporiser	MSS International Ltd.
Oxygen Concentrator	Bitmos Medizintechnik
Stereotaxic frame	Kopf
Surgical Drapes	Medray
Blades No. 13	VWR International
Cotton Buds	VWR International
Heating pad	Beurer
Betadine	Comparative Medicine TCD
EMLA Cream	Comparative Medicine TCD
Xylocaine Spray (lidocaine)	Comparative Medicine TCD
LACRI-LUBE Eye ointment	Comparative Medicine TCD
Buprenorphine	Comparative Medicine TCD
Vaseline	Boots Ireland
Micro Drill	Dremel
Hamilton ® Neuros (7002 KH SYR) syringe	Hamilton, Switzerland
Replacement needles (Neuros syringe NRS75 5.0µl (33/20/3)	Hamilton, Switzerland
Tissue Adhesive (Surgibond)	Comparative Medicine TCD

Behavioural Testing

Material	Supplier
Staircase (model 80300)	Campden Instruments LTD.
Panasonic Camcorder (HC-V250)	Currys PC World
B/W USB camera	Ugo Basile
Canon Camera	Canon
Glass Cylinder (20cm diameter x 50 cm high)	
Plastic Cylinder (29cm diameter x 44cm height)	
Plastic basin (50cm diameter x 39cm high)	Woodies
Open Field (1 metre diameter)	
Syringe needles (G and mm here)	Comparative Medicine TCD
Syringes, plastic (1ml)	Comparative Medicine TCD
d-amphetamine	Sigma-Aldrich

Immunohistochemistry

Material	Supplier
3,3'-diaminobenzidine (DAB)	Sigma-Aldrich
Anti-tyrosine hydroxylase (MAB318; monoclonal mouse)	Millipore
Anti-alpha-synuclein (AB5038; polyclonal rabbit)	Millipore
Elite Vectastain® ABC kit (containing Mouse IgG secondary antibody) (PK-6100)	Vector Laboratories
Alexa488 goat anti-rabbit (A11008)	Bio-Sciences Ltd
CF660 goat anti-mouse (SAB4600451)	Merck
CF594 goat anti-mouse (SAB4600321)	Merck
DPX mounting medium	Fisher Scientific
DABCO PVA mounting medium	Sigma-Aldrich
Hydrogen peroxide (30%)	Sigma-Aldrich
Normal Goat Serum (NGS)	Sigma-Aldrich
Normal Horse Serum (NHS)	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Xylenes	HAZMAT TCD

2.2 Methods

2.2.1 Animals

Male and female Wistar Han rats, aged 7-8 weeks (220-250g male; 200-220g female) are obtained from Envigo, UK, or Comparative Medicine, Trinity College Dublin (TCD), and housed in pairs in individually ventilated cages. Animals are kept in climate controlled rooms set to 21°C (plus or minus 2°C) with relative humidity levels of 50% on a 12:12 hour light/dark cycle. The experimental protocols used are in accordance with the European directive 2010/63/EU on the protection of animals used for scientific purposes and all experimental procedures are approved by the Animal Research Ethics Committee in TCD and performed under the licence granted by the Health Products Regulatory Authority (HPRA).

2.2.2 Adeno-Associated Viral (AAV) Vectors

The AAV vectors for the wildtype (WT) studies are obtained from the Viral Vector Core of the University of Iowa, United States and are serotype AAV5. The constructs of the AAV vectors are outlined in Figure 2.1 below. Briefly, they are composed of a cytomegalovirus (CMV) enhancer and a chicken beta-actin (CBA) promoter. The genetic inserts are enhanced Green Fluorescent Protein (eGFP) or WT human alpha-synuclein. The final viral titres for the AAV5-eGFP and AAV5-alpha-synuclein are 9.5×10^{12} vg/ml and 1×10^{13} vg/ml respectively. Empty vector is also included (AAV2/5-CBA-empty; 2.2×10^{13} vg/ml) as control.

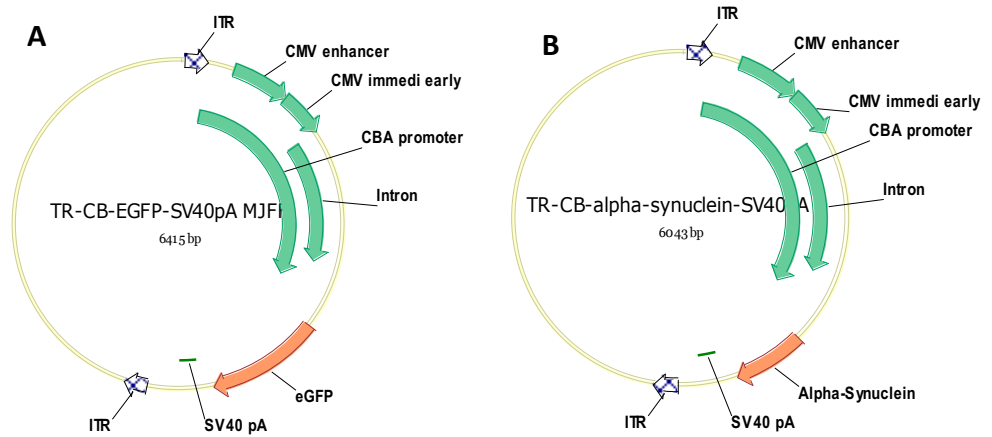


Figure 2.1: Shuttle plasmid maps for (A) AAV5-CBA-eGFP and (B) AAV5-CBA-alpha-synuclein (human WT).

In the A53T studies the serotype of the AAV is AAV1/2. Three different AAV1/2 vectors are used: AAV1/2-CMV-A53T alpha-synuclein human (5.1×10^{12} vg/ml), AAV1/2-CMV-Null/Empty (5.1×10^{12} vg/ml), and AAV1/2-CAG-miR scrambled control alpha-synuclein co-expressing eGFP (0.5×10^{12} vg/ml). These AAV vectors are obtained from Vigene Biosciences, Maryland, USA. They consist of a CMV promoter (A53T alpha-synuclein and Null-Empty) or chicken beta-actin cytomegalovirus hybrid (CAG) promoter (scrambled control), Woodchuck hepatitis virus Posttranscriptional Regulatory Element (WPRE), Bovine Growth Hormone (BGH) and polyadenylation sequence. A CAG promoter consists of the CMV enhancer fused to the CBA promoter. The addition of WPRE, BGH and a polyadenylation sequence is adopted in order to encourage the transcription of the viral nuclear material carried by the AAV vector. The WPRE element has been found to increase transgene expression, however the exact mechanism through which it achieves this is not fully understood. The AAV1/2-CAG-miR scrambled control alpha-synuclein co-expressing eGFP is used in these studies to investigate the impact of the expression of eGFP and it is not used as a direct comparison to the other AAV vectors due to the presence of the miR scrambled control alpha-synuclein which makes it incomparable.

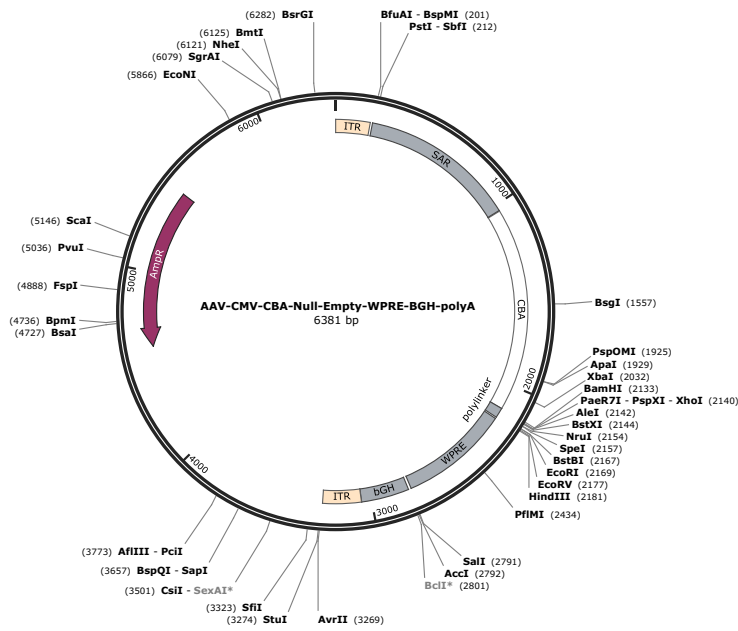


Figure 2.2: Shuttle plasmid map for AAV1/2-CMV-Null/Empty.

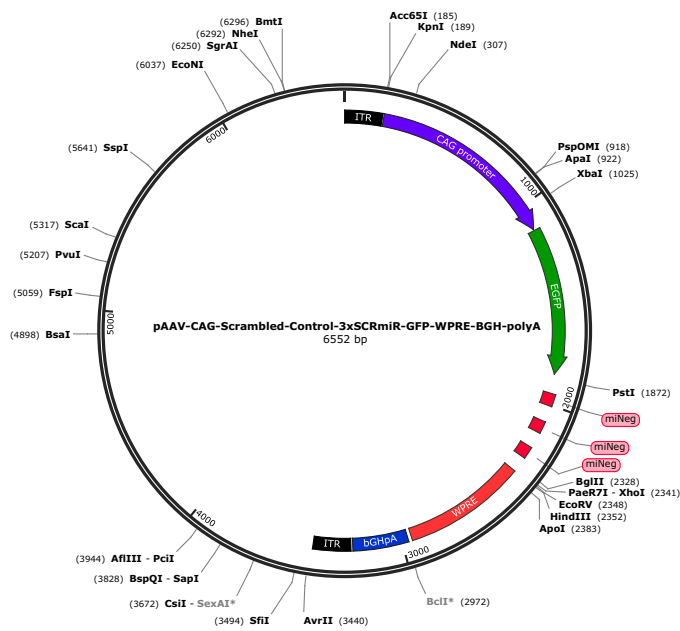


Figure 2.3: Shuttle plasmid map for AAV1/2-CAG-miR scrambled control alpha synuclein co-expressing eGFP.

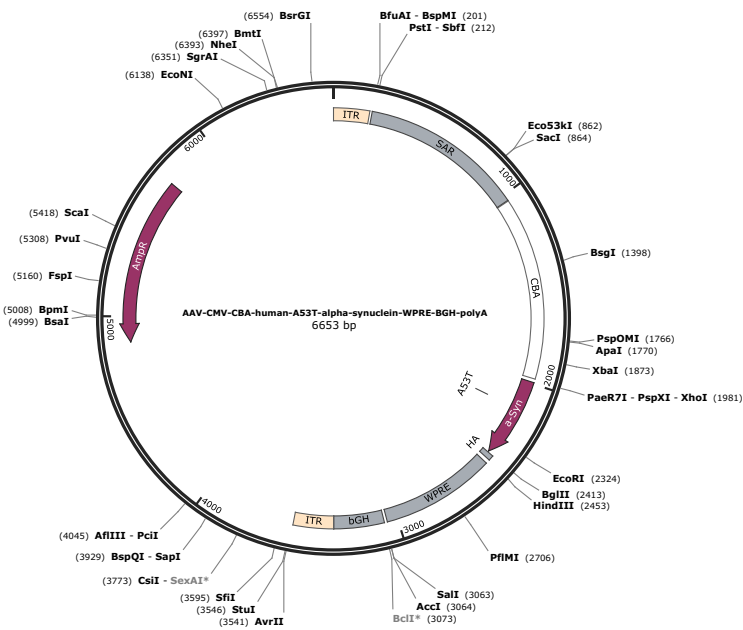


Figure 2.4: Shuttle plasmid map for AAV1/2-CMV-A53T alpha-synuclein human.

2.2.3 Pre-Formed Fibrils (PFFs)

Pre-formed fibrils are obtained from StressMarq Biosciences, Victoria, British Columbia, Canada. Two different PFFs are used in this experiment: Type 1 PFFs Human Wildtype alpha-synuclein (2µg per 1µl in Phosphate Buffered Saline (PBS); SPR-322) and Type 1 PFFs Human A53T alpha-synuclein (2µg per 1µl in PBS; SPR-326). The PFFs are stored at -80° Celsius until use.

2.2.3.1 Preparation of PFFs

The PFFs are thawed to room temperature and sonicated for 6 sets of 10 x 0.5 second pulses, leaving a 10 second pause between each set of pulses to prevent heat generation and possible protein denaturation. PFFs are then aliquoted in order to prevent exposing the PFFs to multiple freeze thaw cycles before use in surgery.

2.2.4 Stereotaxic Surgery

Surgical procedures are performed using aseptic technique under isoflurane anaesthesia, with induction at 5% and maintenance at 2% in 1.5L/min oxygen (O₂). Briefly, anaesthesia is induced, the rats heads are shaved and the rats are then placed in the stereotaxic frame. LACRI-LUBE or Vaseline is placed over the eyes to protect them from drying out. Betadine and lidocaine are applied to the skin and a small midline incision is made. The connective tissue is removed and bregma is located. Using bregma on the skull as reference a burr hole is drilled in the skull at the location specified in the figures below. A Hamilton® Neuros syringe (7002 KH SYR; Hamilton, Switzerland) with a 33 gauge removable needle is used for all injections, with the injected solution being delivered at a rate of 1µl per minute. Following injection, the needle is left in place for a further 4 minutes and then slowly removed over 1-2 minutes to insure diffusion and prevent efflux up the needle tract. Following surgery the incision is closed with a surgical adhesive. Rats are individually housed following surgery to aid recovery and until normal behaviour had returned at which time they are re-housed with their cage mate. The time from the initial anaesthesia to procedure completion is kept as short as possible.

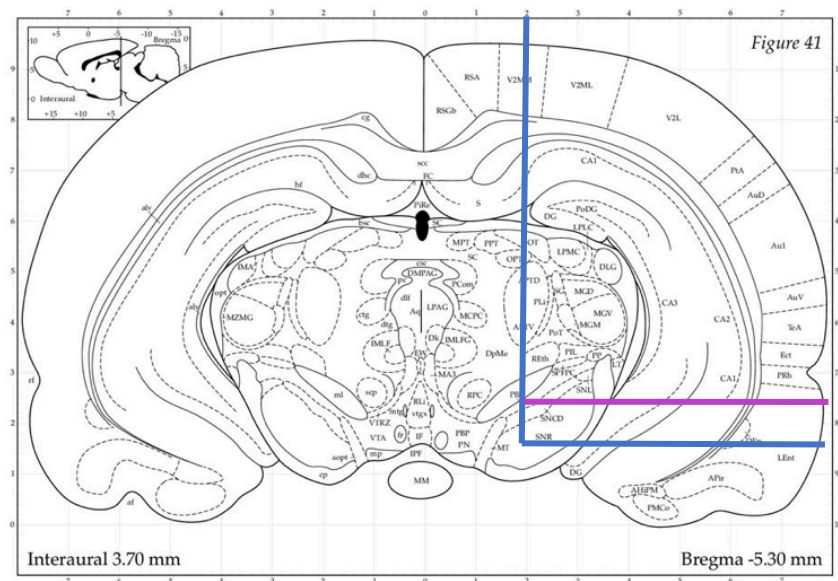


Figure 2.5: The stereotaxic coordinates for unilateral injection into the substantia nigra pars compacta for all AAV injections. As the injections for dorsal-ventral (DV) are from the skull the DV line is for illustrative purposes only and is not exact. Blue medial-lateral (ML) line represents injection co-ordinates for males throughout the studies, and for the females in the first study. Pink line represents ML injection co-ordinates for the females following adaptation of protocol. Male co-ordinates: anterior-posterior (AP) AP -5.3mm , ML $+2.0\text{mm}$, DV -8.5mm ; Female co-ordinates AP -5.3mm , ML $+2.0\text{mm}$, DV -7.5mm . Image adapted from Paxinos and Watson Rat Brain Atlas (1982).

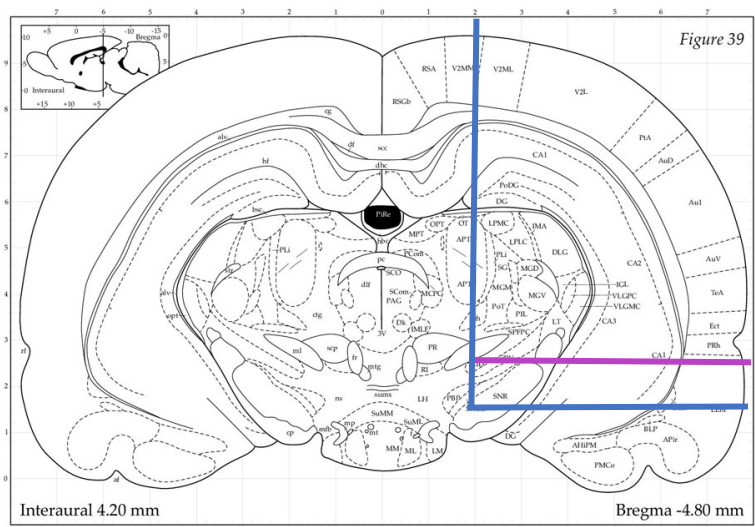


Figure 2.6: The stereotaxic coordinates for unilateral injection into the substantia nigra pars compacta for all PFF injections. As the injections for DV are from the skull the DV line is for illustrative purposes only and is not exact. AP co-ordinates are -4.9mm, however this is not available as an image in Paxinos and Watson Rat Brain Atlas, therefore the AP co-ordinate presented is not exact. Blue ML line represents injection co-ordinates for males and pink line represents ML injection co-ordinates for the females. Male co-ordinates AP – 4.9mm, ML +2.0mm, DV –8.5mm, female co-ordinates AP –4.9mm, ML +2.0mm, DV –7.5mm. Image adapted from Paxinos and Watson Rat Brain Atlas (1982).

2.2.5 Behavioural Testing

All behavioural testing, with the exception of the open field test, are unilateral motor behavioural tests. These unilateral tests are employed as the intranigral injection utilised in this study is unilateral in nature, allowing for the un-injected hemisphere to be used as control. All behavioural deficits are expected to be on the side contralateral to the injected hemisphere, with the exception of the amphetamine test in which increased ipsilateral rotations would be expected following a unilateral dopaminergic lesion.

2.2.5.1 Staircase Test

The staircase test, developed by (Montoya et al., 1991), is employed to analyse skilled motor performance. The staircase apparatus consists of (1) an entrance chamber (20cm long x 11.5cm wide x 11.5 cm high) with a series of adjacent metal bars for a floor and a clear plexiglass hinged lid, (2) an adjacent reaching chamber (16.5cm long x 6cm wide x 11cm high) made from clear plexiglass, (3) a central platform within the reaching chamber (16.5cm long x 2.5cm wide x 5.5cm high) and (4) a staircase of 7 steps on either side of the platform, each with a shallow groove in the centre of the step. During training and testing, two sugar pellets (Cocopops®) are placed in each groove. As this is a complex task, each rat receives a number of training sessions in the task prior to baseline data collection and surgery. The number of training sessions are specified in each results chapter, as due to optimisation of the test, there are different numbers of training sessions per chapter. During the training sessions, Cocopops® are placed on each step and the rat is given a 10 minute trial in the apparatus.

During behaviour testing the rat is placed in the staircase apparatus for 10 minutes and allowed to retrieve the pellets, with the rats behaviour recorded for later analysis. A 'reaching attempt' is taken as any limb motion which displaces a pellet from its step. Successful reaching attempts are defined as a reaching attempt which leads to successful retrieval and eating of the Cocopop® using the paw. Unsuccessful reaching attempts are defined as a reaching attempt in which the pellet is (1) displaced from the step onto another

step or (2) picked up and dropped onto the bottom of the chamber. The 'success rate' is calculated as the number of successful reaching attempts made with paw expressed as a percentage of the total reaching attempts made with paw.

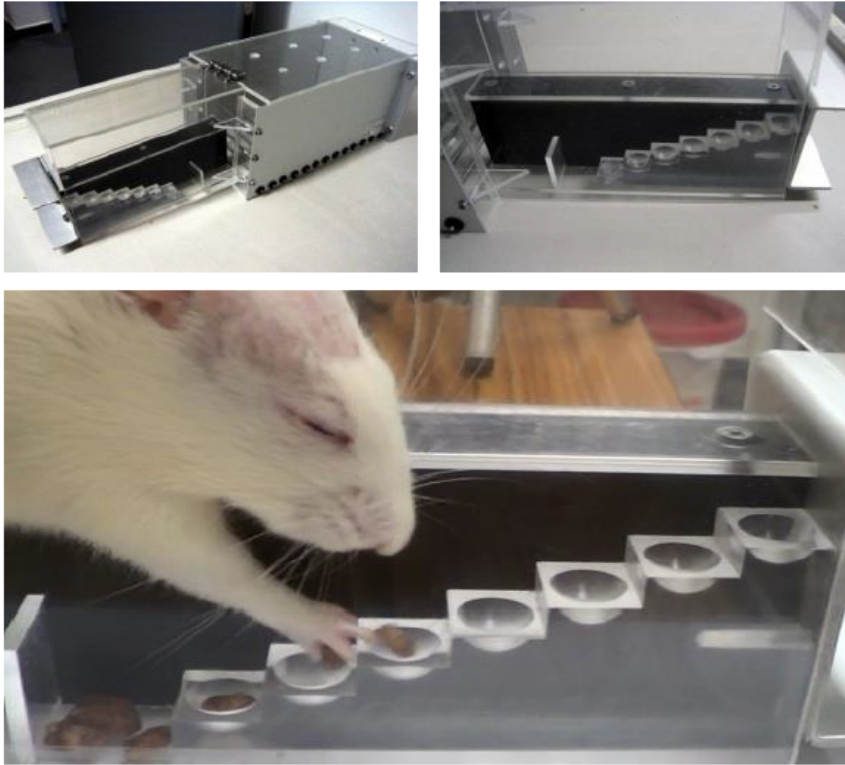


Figure 2.7: Staircase test of skilled motor function. Rats are placed in this apparatus for ten minutes. Their behaviour is video recorded and the contralateral limb success rate is expressed as the total number of successful reaching attempts divided by the total number of attempts and expressed as a percentage.

Scoring of the Staircase Test

Criterion	Description
Successful reaching attempt	Pellet is picked up with paw and retrieved.
Unsuccessful reaching attempt 1	Pellet is picked up and then dropped.
Unsuccessful reaching attempt 2	Pellet is intentionally reached for and knocked from its step onto another step, onto the floor of the apparatus or into the chamber.
Retrieved with tongue	The pellet is retrieved with the tongue and not the paw.
Retrieved from chamber	The pellet is knocked from a step, into the chamber, and retrieved from within the chamber by the rat.
Total Fails	(unsuccessful reaching attempt 1 + unsuccessful reaching attempt 2).
Total Successes	Successful reaching attempt – (Total fails + retrieved with tongue + retrieved from chamber).
Success rate	$(\text{Total Successes} / \text{Total pellets at start of trial}) * 100$

Table 2.1: Criteria for scoring of the staircase test.

Not all animals successfully acquire the staircase test at baseline testing. In order to allow any behaviour differences to be identified due to treatment and not just poor performance at baseline two exclusion criteria are adopted following baseline performance, with these animals being excluded from all analysis.

Criterion	Description
60/40 right/left or left/right performance	60/40 preference at baseline for one paw over the other.
< 4 pellets retrieved on either right of left side	Pellets retrieved at baseline less than 4 pellets as this was below the 25% interquartile range for total number of pellets baited per side of the apparatus.

Table 2.2: Exclusion criteria for the staircase test.

2.2.5.2 Stepping Test

The stepping test is used as a test of forelimb akinesia and is performed as previously described by (Olsson et al., 1995). Animals are restrained in the investigators hand with one limb remaining free. The free forelimb is placed on a surface, which is one metre in length, and the animal is moved in a forward direction in a wheelbarrow-like motion over approximately five seconds. The is repeated in the backward direction. The number of adjusting steps making contact with the table top is recorded in both the forward and backward directions for each forelimb. The number of steps made by the contralateral limb is expressed as a percentage of the total number of steps made by both limbs in that direction.

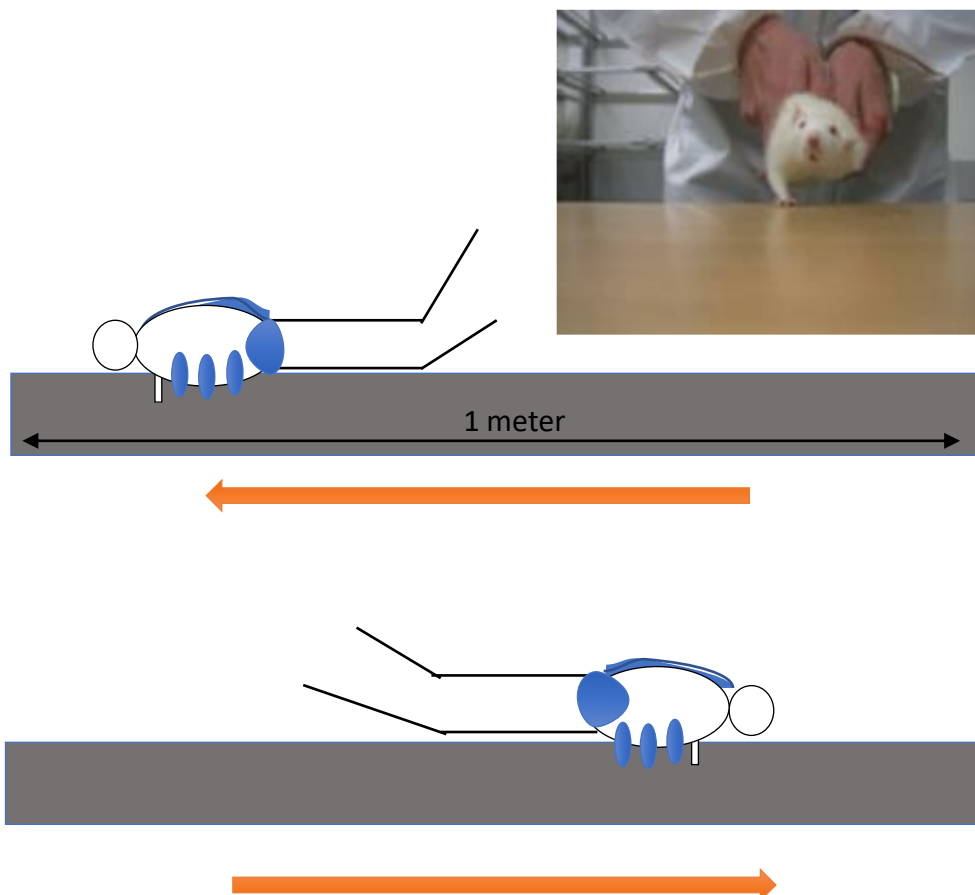


Figure 2.8: Illustration of the performance of the stepping test. Three of the four limbs of the animal are restrained and the animal is then guided forwards and backwards along a surface which is 1 meter in length. The number of adjusting steps made by the animal is recorded.

Data Analysis

The data collected over the three trials are analysed in three different ways: average, median and maximum. Following review of the data and the literature (Becker et al., 2021, Bourdenx et al., 2015) it was decided that all data be presented using the average where three trials were performed.

Analysis is initially performed using the raw data collected. Following this analysis data are analysed as percentages.

% contralateral forward steps = number of contralateral forward steps/(number of contralateral forward steps + number of ipsilateral forward steps)*100

% contralateral backward steps = number of contralateral backward steps/(number of contralateral backward steps + number of ipsilateral backward steps)*100

% ipsilateral forward steps = number of ipsilateral forward steps/(number of ipsilateral forward steps + number of contralateral forward steps)*100

% ipsilateral backward steps = number of ipsilateral backward steps/(number of ipsilateral backward steps + number of contralateral backward steps)*100

Data are analysed as change from baseline, both raw change and percentage change. This data is not presented as an overall drop in number of steps is observed across treatment groups following repeated testing. Baseline data is used to confirm that there are no differences between the groups pre-surgery. Post-surgical data compares both the raw data for contralateral and ipsilateral, and the data expressed as % contralateral and % ipsilateral within and across groups. This method of data analysis is used as all surgeries performed are unilateral, which allows for each animal to act as their own control.

2.2.5.3 Cylinder Test

The cylinder test is used to evaluate limb-use asymmetry following unilateral damage to the nigro-striatal tract and is performed as previously described by (Schallert et al., 2000). Rats are placed in a clear cylinder for five minutes and recorded with a camcorder. When placed in a novel environment, such as a cylinder, rats engage in exploratory behaviour and rear, making contact with the wall of the cylinder. Video analysis records the number of wall placements upon rearing, and which limbs make contact with the wall. Each placement is recorded as either 'left', 'right' or 'simultaneous' if both paws make contact at the same time. The cumulative number of wall placements for each limb is expressed as the number of total wall placements.

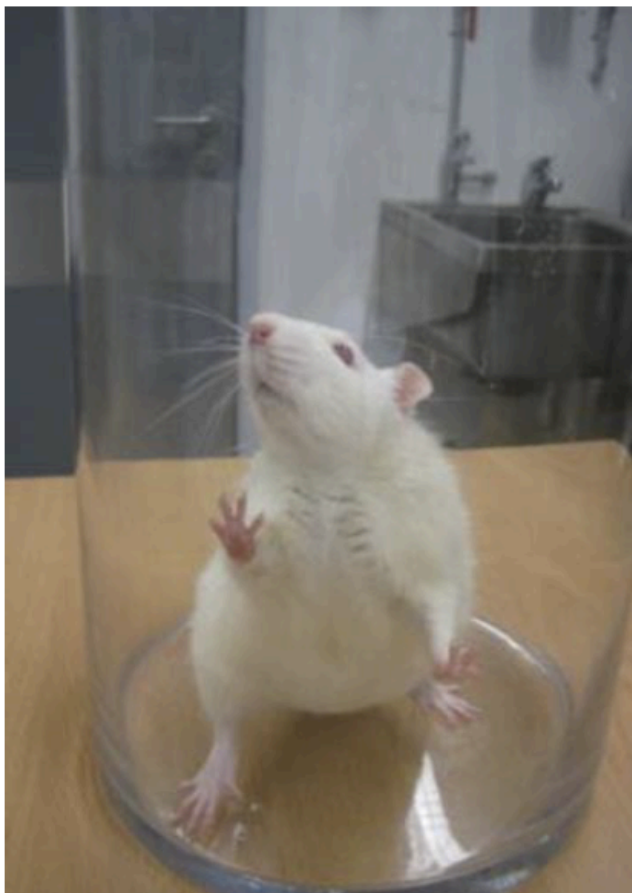


Figure 2.9: The cylinder test of limb use asymmetry. The rats are placed in the cylinder for a period of five minutes and recorded from above. The total number of contacts made by each limb is recorded and the contralateral limb usage is divided by the total number of limb contacts and expressed as a percentage.

Data Analysis

For all studies recorded with a camera placed above, wall placements are scored. A wall placement is defined as a movement in which an animal rears and places one or both of their limbs on the wall of the cylinder. The first limb to make contact upon rearing is counted, with any subsequent wall placements also scored. These can be right limb, left limb, or simultaneous, in which both limbs make contact with the wall at the same time. If the animal then places the other limb on the wall this is scored as simultaneous. During exploration of the cylinder wall the animal may engage in wall stepping, in which they use both paws to support themselves as they move around the cylinder. Each time the animal uses one paw and then the other in this wall stepping motion a score of one is given to simultaneous wall contacts. If the animal repeatedly makes contact with the wall of the cylinder with one limb, a score for each contact is given to that limb.

For the studies in which the animals behaviour is recorded from both sides, the scoring of the wall placements remains as outlined for the studies recorded from above. The additional parameter of floor landing is included in the analysis of these studies. This counts the paw which lands on the floor first following a rear. This can be left, right or simultaneous. In contrast to the wall placements, in which all wall placements are counted, only the first floor landing is counted. Any exploration following this initial landing is not counted.

Analysis for all studies is initially performed using the raw data collected. Following this analysis data are also analysed as percentages.

% left wall placements = $\text{left wall placements} / (\text{left} + \text{right} + \text{simultaneous wall placements}) * 100$

% right wall placements = $\text{right wall placements} / (\text{left} + \text{right} + \text{simultaneous wall placements}) * 100$

% simultaneous wall placements = simultaneous wall placements/(left + right + simultaneous wall placements)*100

% left floor landings = left floor landings/(left + right + simultaneous floor landings)*100

% right floor landings = right floor landings/(left + right + simultaneous floor landings)*100

% simultaneous floor landings = simultaneous floor landings/(left + right + simultaneous floor landings)*100

Additional analysis parameters are also included, in the form of limb use asymmetry scores which are calculated as follows:

Limb Use Asymmetry Score Wall = % unimpaired limb wall placements - % impaired limb wall placements

Limb Use Asymmetry Score Floor = % unimpaired limb floor landings - % impaired limb floor landings

Limb Use Asymmetry Score = (% unimpaired limb wall placements + % unimpaired limb floor landings) – (% impaired limb wall placements + % impaired limb floor landings)

2.2.5.4 Amphetamine-induced rotation test

The amphetamine-induced rotation test is used as a measure of the extent of motor impairment that is present following a unilateral lesion to dopaminergic cells. The rats are placed in single open top cages with bedding and allowed to habituate to the cage for 20 minutes. Rats are then injected with d-amphetamine (5mg/kg, s.c.) and returned to the cages, where they are recorded using a camcorder for a period of time, ranging from 1 hour to 2 hours. Due to optimisation of the test, different test times were used throughout the project and these times are illustrated in each results chapter. These videos are scored for ipsilateral and contralateral rotations, starting five minutes post-injection. Results are expressed as net ipsilateral and net contralateral rotations per minute, as well as total ipsilateral rotations and total contralateral rotations.



Figure 2.10: The amphetamine rotation test. The rats are administered amphetamine (5mg.kg s.c.) and singly housed for an hour, during which time their behaviour is video recorded. The number of ipsilateral rotations is counted and expressed as number of rotations per minute, as well as total ipsilateral rotations.

2.2.6 Immunohistochemistry

2.2.6.1 Tissue Processing

Rats are deeply anaesthetised with isopentane or sodium pentobarbital and transcardially perfused with Phosphate Buffered Saline (PBS) followed by 4% paraformaldehyde (PFA). Once perfusion is complete, the animals are decapitated, brains removed and stored overnight in 4% PFA (4°C) before being cryoprotected in 30% sucrose solution for 72 hours (4°C) or until the brains sink to the bottom of the tubes. Brains are then snap frozen in isopentane on dry ice and stored at -80°C. 35µm coronal sections through the substantia nigra are cut using a cryostat. These sections are placed into collection tubes containing freezing solution (75g sucrose, 75ml ethylene glycol made up to 250ml with PBS). Five collection tubes mean that every serial selection is spaced by 175 µm with 10 sections/collection tube allowing for appropriate stereological analysis.

2.2.6.2 Free floating immunohistochemistry

The primary antibody utilised for alpha-synuclein (AB5038; polyclonal rabbit; Millipore) is not specific to A53T alpha-synuclein, and is reactive for both human and rat alpha-synuclein. This antibody is used throughout all studies, including when AAV-A53T alpha-synuclein and PFF A53T alpha-synuclein is intranigrally injected.

Sections are washed in PBS 0.1% Triton three times for ten minutes each in between each step as outlined below up to the secondary antibody incubation. The sections are incubated for twenty minutes in 3% hydrogen peroxide (H₂O₂) in 10% methanol (MeOH) PBS. The sections are then blocked and treated with primary and secondary antibodies as outlined in Table 2.3.

Blocking agent	Primary Antibody	Dilution	Secondary Antibody	Dilution
3% normal horse serum in 0.1% PBS-triton 45 minutes	Mouse anti-tyrosine hydroxylase (MAB318; monoclonal) in 1% NHS in 0.1% PBS-triton. Overnight	1:2000	Biotinylated horse anti-mouse in PBS (Elite Vectastain© ABC kit (containing Mouse IgG secondary antibody) (PK-6100)). 90 minutes	1:200

Table 2.3: Blocking agents and antibody dilutions for free-floating immunohistochemistry.

Following secondary antibody incubation the sections are washed three times with PBS for 10 minutes each. The sections are then incubated with the avidin biotin complex (ABC) for 90 minutes to visualise the staining, using peroxidase as the enzyme, H_2O_2 as the substrate and 3,3-Diaminobenzidine (DAB) as the chromogen. The DAB reaction is started by adding 1ml of DAB to 20ml of PBS, and just prior to commencing the reaction, 6 μ l of 30% H_2O_2 . The DAB reaction is stopped with distilled H_2O and the sections are mounted on gelatin-coated slides and left to dry overnight at room temperature. The slides are then desalted in distilled H_2O for 2 minutes, followed by dehydration in a series of ascending alcohols: 70% Ethanol (EtOH) $\rightarrow$ 90%EtOH $\rightarrow$ 100%EtOH for two minutes each and then cleared in Xylene for one minute. Using DPX Mounting Media the slides are cover-slipped and left to dry overnight.

2.2.6.3 Sections directly mounted on the slide immunohistochemistry

Slides are washed in PBS 0.1% Triton three times for ten minutes each in between each step as outlined below up to the secondary antibody incubation. The sections are incubated for ten minutes in 3% H_2O_2 in MeOH. The slides are then blocked and treated with primary and secondary antibodies as outlined in Table 2.4.

Blocking agent	Primary Antibody	Dilution	Secondary Antibody	Dilution
3% normal horse serum in 0.1% PBS-triton. 1 hour	Mouse anti-tyrosine hydroxylase (MAB318; monoclonal) in 1% NHS in 0.1% PBS-triton. Overnight	1:2000	Biotinylated horse anti-mouse in PBS (Elite Vectastain© ABC kit (containing Mouse IgG secondary antibody) (PK-6100)). 2 hours	1:200

Table 2.4: Blocking agents and antibody dilutions for free-floating immunohistochemistry.

Following secondary antibody incubation the sections are washed three times with PBS for 10 minutes each. The sections are then incubated with the ABC for 2 hours to visualise the staining, using peroxidase as the enzyme, hydrogen peroxide as the substrate and DAB as the chromogen. The DAB reaction is started by adding 1ml of DAB to 20ml of PBS, and just prior to commencing the reaction, 6µl of 30% H₂O₂. The DAB reaction is stopped with distilled H₂O the slides are then desalted in distilled H₂O for 2 minutes, followed by dehydration in a series of ascending alcohols: 70%EtOH -> 90%EtOH -> 100%EtOH for 2 minutes each and then cleared twice in Xylene for two minutes each. Using DPX Mounting Media the slides are coverslipped and left to dry overnight.

2.2.7 Immunofluorescence

2.2.7.1 Free floating immunofluorescence

Sections are washed in PBS 0.1% Triton three times for ten minutes each between each step as outlined below. The sections are blocked and treated with primary and secondary antibodies as outlined in Table 2.5.

Blocking Agent	Primary Antibody	Dilution	Secondary Antibody	Dilution
3% normal goat serum in PBS-triton 0.1%. 2 hours	Mouse anti-tyrosine hydroxylase (MAB318; monoclonal) in PBS-triton 0.1%. Overnight	1:2000	CF660 goat anti-mouse (SAB4600451) OR CF594 goat anti-mouse (SAB4600321) in PBS-triton 01%. 2 hours	1:500
3% normal goat serum in PBS-triton 0.1%. 2 hours	Anti-alpha-synuclein (AB5038; polyclonal rabbit) in PBS-triton 0.1%. Overnight	1:2000	Alexa488 goat anti-rabbit (A11008) in PBS-triton 0.1%. 2 hours	1:500

Table 2.5: Blocking agents and antibody dilutions for free-floating immunofluorescence.

Following the secondary antibody incubation the sections are washed 3 times in PBS for ten minutes each. The sections are then incubated with 4',6-diamidino-2-phenylindole (DAPI) (1:5000 in distilled H₂O) for five minutes. DAPI is a fluorescent stain that binds strongly to adenine–thymine-rich regions in DNA and is commonly used to stain the nucleus of cells. This is followed by two ten minute PBS washes, after which the sections are mounted onto gelatin-coated slides and cover-slipped using DABCO PVA Mounting Media and sealed with nail varnish.

2.2.7.2 Sections directly mounted on the slide immunofluorescence

Slides are washed in PBS 0.1% Triton three times for ten minutes each between each step as outlined below. The sections are blocked and treated with primary and secondary antibodies as outlined in Table 2.6.

Blocking Agent	Primary Antibody	Dilution	Secondary Antibody	Dilution
3% normal goat serum in PBS-triton 0.1%. 2 hours	Mouse anti-tyrosine hydroxylase (MAB318; monoclonal) in PBS-triton 0.1%. Overnight	1:2000	CF660 goat anti-mouse (SAB4600451) OR CF594 goat anti-mouse (SAB4600321) in PBS-triton 0.1%. 2 hours	1:500
3% normal goat serum in PBS-triton 0.1%. 2 hours	Anti-alpha-synuclein (AB5038; polyclonal rabbit) in PBS-triton 0.1%. Overnight	1:2000	Alexa488 goat anti-rabbit (A11008) in PBS-triton 0.1%. 2 hours	1:500

Table 2.6: Blocking agents and antibody dilutions for immunofluorescence.

Following the secondary antibody incubation the slides are washed 3 times in PBS for ten minutes each. The sections are then incubated with DAPI (1:5000 in distilled H₂O) for five minutes. This is followed by two ten minute PBS washes, after which the sections are cover-slipped using DABCO PVA Mounting Media and sealed with nail varnish.

2.2.8 Image Analysis

2.2.8.1 Immunohistochemistry

Sections stained using the ABC and DAB visualisation process are imaged using an Olympus DRP72 camera mounted on an Olympus BX51 normal light microscope and analysed using Image J software (Image J v1.48x; NIH, US). The number of tyrosine hydroxylase positive cells are manually counted using the cell counter tool in ImageJ.

Two-dimensional images are acquired of a region of the substantia nigra at 40x magnification. The number of cells visible in these images are then manually counted using the ImageJ cell counting tool (Image J v1.48x; NIH, US). The

region of the substantia nigra imaged is maintain constant across hemispheres, sections and animals. A minimum of three sections of the substantia nigra are assessed per animal. This method of analysis is not unbiased stereology and therefore the cell numbers obtained are not absolute cell numbers. This method is used in order to assess potential cell loss, while understanding the limitation of this approach in that it does not allow for absolute cell numbers to be quantified.

2.2.8.2 Immunofluorescence

Immunofluorescent sections are imaged using an Olympus Ix81 fluorescence microscope and an Andor iXon+ ultra-sensitive EMCCD camera or an AxioImager Z1 epifluorescent microscope with a Zeiss AxioCam HR camera.

Image analysis protocol 1: Images of the substantia nigra are acquired at 20X magnification. Staining intensity of the substantia nigra for alpha-synuclein is measured using the Analyse -> Measure function on ImageJ. From this the mean pixels value is generated. This is then expressed as a percentage of the un-injected side, as well as being expressed as arbitrary units.

Image analysis protocol 2: Images of the substantia nigra are acquired at 20X magnification. The images are converted to an 8-bit image using ImageJ. Using the threshold option in ImageJ the histogram of all the pixel intensities is generated. A threshold is then set which removes most of the background staining in the image (Figure 2.11). This is achieved by starting the threshold where the histogram begins to taper off towards a flat line and includes all value past this initial tapering off point. The Analyse -> Measure function is then utilised on Image J. From this the mean pixels value/arbitrary units value is generated. This is then expressed as a percentage of the un-injected side, as well as being expressed as arbitrary units.

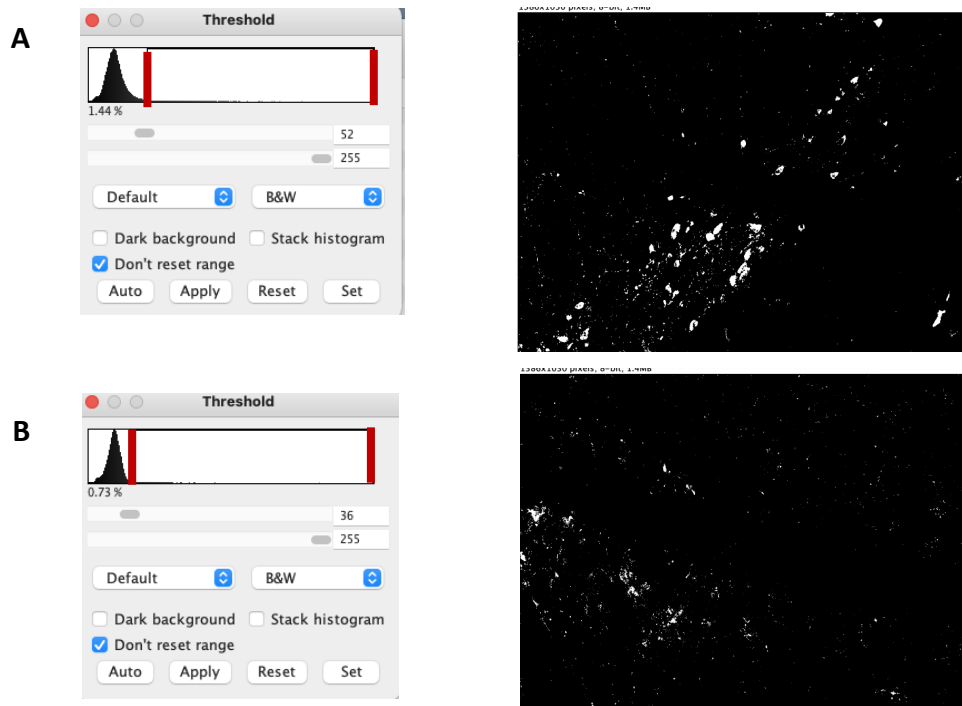


Figure 2.11: Example of threshold setting for the immunofluorescence analysis for image analysis protocol 2. A) Setting the threshold for the injected hemisphere at the point at which the histogram levels off. Redlines indicate the threshold used. B) Setting the threshold for the un-injected hemisphere at the point at which the histogram levels off.

2.2.9 Statistical Analysis

As these studies include both males and females, the data are first analysed by treatment group, in which the male and female data are combined together. Data are then analysed for male and female separately, using the same analysis techniques applied to both sexes combined.

Data are analysed using GraphPad Prism 8 software. In the first instance, data are analysed using Shapiro-Wilk test for normality. Depending on whether the data were normally or not normally distributed the following statistical tests are applied.

2.2.9.1 Behavioural Data

Behavioural data are analysed using a two-way ANOVA or a one-way ANOVA followed by Neuman-Keuls multiple comparisons tests where appropriate, or an unpaired t-test.

Behavioural data comparing three or more data sets which was found to be not normally distributed are analysed using Kruskal-Wallis test followed by Dunn's multiple comparisons test where appropriate.

A Brown-Forsythe ANOVA is employed when comparing three or more data sets which were found to not have equal variances, followed Dunnett's T3 multiple comparisons test where appropriate.

An unpaired t-test with Welches correction is used when two data sets were compared that were found to not have equal variances.

A Mann Whitney test is employed when the data sets were compared which were found to not have a normal distribution.

2.2.9.2 Immunohistochemistry and Immunofluorescence Data

Immunohistochemical data is analysed using one-way ANOVA followed by Neumann-Keuls multiple comparisons tests where appropriate, an unpaired t-test or a paired t-test.

When three or more data sets were compared, which are found to not have a normal distribution a Kruskal-Wallis test is employed, followed by Dunn's multiple comparisons test, where appropriate.

When paired data are found not to have a normal distribution, a Wilcoxon matched-pairs signed rank test is used.

When two data sets are analysed which are found to have different variances, an unpaired t-test with Welch's correction is used.

$p < 0.05$ is considered statistically significant. Data are expressed as mean with standard error of the mean (SEM) for normally distributed data and median with interquartile range, if data are not normally distributed.

3. Assessing the implications of expression of wildtype human alpha-synuclein in the rat substantia nigra through the use of adeno-associated viral vectors

3.1 Introduction

The accumulation of alpha-synuclein is a key pathological hallmark of Parkinson's disease (PD), which leads to the development of the proteinaceous cytoplasmic inclusions known as Lewy bodies and Lewy neurites (Jakes et al., 1994). A pathogenic role for alpha-synuclein is supported by genetic data from various studies assessing the implications of multiplications of the *SNCA* gene, which encodes alpha-synuclein, as well as various point mutations and polymorphisms of the gene (Chartier-Harlin et al., 2004, R Krüger, 1998, Polymeropoulos et al., 1997, Zarranz, 2003). In order for the levels of alpha-synuclein to remain in a functional state in the central nervous system (CNS) there must be a balance between the rates of synthesis, aggregation and clearance (Kragh et al., 2012). Disruption of this balance can lead to atypical levels of alpha-synuclein. It is thought that a polymorphism occurring at the promoter region of *SNCA*, known as Rep1, can lead to increased risk of developing PD through increasing the expression of alpha-synuclein (Maraganore et al., 2006).

The use of viral vectors has become an attractive way to express transgenes and develop novel rodent models of disease. Through direct targeting of the substantia nigra, transgenes of interest can be expressed in adult rodents, and high expression levels can be utilised. Much research has been conducted utilising wildtype (WT) alpha-synuclein expression through the use of adeno-associated viral (AAV) vectors in order to develop preclinical models of PD, as illustrated in Table 1.2 in Chapter 1. Alpha-synuclein expression has been

confirmed following intranigral injection of AAV WT human alpha-synuclein at multiple timepoints post-delivery. For example, rAAV2/5 delivery of human WT alpha-synuclein in the substantia nigra of female Sprague Dawley rats leads to alpha-synuclein positive cell expression in the substantia nigra at four weeks post-AAV delivery, and this expression was found to continue up to the end timepoint of the study, at 15 weeks post-AAV delivery (Sanchez-Guajardo et al., 2010). The development of alpha-synuclein positive aggregates have also been reported following AAV mediated delivery of human WT alpha-synuclein to the rodent substantia nigra (Gorbatyuk et al., 2008), with some studies reporting that these aggregates are proteinase K resistant (Taschenberger et al., 2012). Kirik and colleagues (2002) report the development of alpha-synuclein inclusions and dystrophic neurites similar to those observed in idiopathic PD at 8 weeks post intranigral AAV-WT-alpha-synuclein delivery.

Tyrosine hydroxylase cell loss is used as a key marker of dopaminergic neuronal degeneration in preclinical models of PD. Intranigral delivery of AAV2-human-WT-alpha-synuclein in female rats has been found to lead to tyrosine hydroxylase (TH) cell loss as early as two weeks post-AAV delivery (Taschenberger et al., 2012), with other studies reporting TH loss following intranigral delivery at three (Kirik et al., 2002), four (Sanchez-Guajardo et al., 2010), six (McFarland et al., 2009a), and eight (Gorbatyuk et al., 2008) weeks post-AAV delivery. The variability between the timepoints at which TH cell loss becomes apparent in these studies could be attributed to the fact that different serotypes, enhancers and promoters were used across the different studies, as illustrated in Table 1.2.

A variety of motor behavioural tests have been utilised by researchers in order to characterise the motor phenotype which develops following AAV mediated delivery of WT alpha-synuclein to the rat substantia nigra. The amphetamine and apomorphine induced rotation tests are commonly employed in order to assess if there is a unilateral dopaminergic lesion present in animals which received a unilateral intranigral injection. However, assessment of behavioural changes following AAV-WT-alpha-synuclein intranigral delivery in the

amphetamine induced rotation test have not provided any clear phenotype. While some studies report the emergence of behavioural deficits in the amphetamine rotation test at 8 weeks post-AAV delivery (Decressac et al., 2011; Decressac et al., 2012a), other studies report no effects in the amphetamine rotation test at 12 weeks post-AAV delivery (Gaugler et al., 2012). While the studies mentioned here all used the serotype AAV2/6, the titres used were different, with injection volumes and titres of 3-4.5µl 3.7×10^{13} genome copies/ml and 3.1×10^8 genome copies/3µl leading to the observed behavioural deficits, and an injection containing a virus dose of 2.8×10^7 transducing units leading to no behavioural deficits.

The stepping test and cylinder test have also been employed by researchers, with differing degrees of behavioural deficits being observed. AAV2/6-human-WT-alpha-synuclein intranigral delivery has been observed to lead to the development of behavioural deficits in the stepping test at three weeks post-AAV delivery, with behavioural deficits only becoming evident at eight weeks post-AAV delivery in the cylinder test (Decressac et al., 2012a). This suggests that perhaps different behavioural tests should be employed at different timepoints post-AAV delivery as motor deficits became evident in the stepping test at an earlier timepoint than the cylinder test.

The differences observed in behavioural tests post-AAV delivery could be down to an number of different factors, including the titre or serotype of the AAV, the enhancers or promoters used. Comparison of two different AAV6 vector constructs, both expressing human WT alpha-synuclein in the substantia nigra of female Sprague Dawley rats aimed to assess the impact of the expression of the gene being driven by the synapsin-1 promoter and enhanced by WPRE compared to the expression of alpha-synuclein under the control of the CBA promoter without a WPRE enhancer (Decressac et al., 2012a). The use of the AAV vector including the WPRE element led to a greater expression of alpha-synuclein in the injected hemisphere of the brain at three weeks post-AAV delivery, even though the titre used to express the alpha-synuclein was the same. Alpha-synuclein expression through the WPRE element led to

behavioural deficits in the cylinder and amphetamine test, with no behavioural impairment was observed in the amphetamine rotation test in the animals injected with alpha-synuclein lacking the WPRE enhancer.

Green fluorescent protein is utilised by many studies to control for the effects of expressing a foreign protein in the rodent brain and also to compare the diffusion of expression of this protein in comparison to alpha-synuclein following intranigral delivery. Intranigral injection of AAV which leads to GFP expression does not lead to the development of alpha-synuclein aggregates (Taschenberger et al., 2012). Furthermore, comparisons of AAV-alpha-synuclein and AAV-GFP injected rodents report TH loss in the AAV-alpha-synuclein, and not the AAV-GFP injected substantia nigra (Sanchez-Guajardo et al., 2010). However, some studies have observed a degree of TH cell loss following AAV-GFP intranigral injection (Gombash et al., 2013, Yamada et al., 2004), with this cell loss being less than that presented by AAV-alpha-synuclein injected animals and not reaching significance.

There is no consensus on the length of time that is needed post-AAV delivery in order to develop a preclinical model of PD. While some researchers report early TH cell loss (Taschenberger et al., 2012) and motor behavioural deficits (Decressac et al., 2012a), other researchers report much longer timeframes, with some studies being performed for 27 weeks post-AAV delivery and failing to elicit motor behavioural impairments (Kirik et al., 2002). Some argue that the variability in the findings relates to the observed differences between animals following intranigral AAV-WT-alpha-synuclein delivery. It has been reported that immunoreactive cell loss can be variable across animals following AAV-WT-alpha-synuclein delivery to the substantia nigra, and can range from 30-40% in some animals to 70-80% in others (Kirik et al., 2002).

Taken together, the studies mentioned above suggest that expression of WT human alpha-synuclein in the rat can be achieved through the injection of AAV vectors. While the results on TH immunoreactive cell loss are variable, and differ across studies, there is a body of evidence suggesting that this cell loss

can be induced through the overexpression of human alpha-synuclein in the rat substantia nigra. The behavioural results are less clear, with some studies showing robust behavioural deficits and others showing none at all. The present study aimed to assess the impact of delivery of human WT alpha-synuclein through AAV5-CBA-alpha-synuclein (human WT) and compare this with the impact of delivery of AAV5-eGFP alone, or AAV5-eGFP and AAV2/5-CBA-Empty, on motor behavioural performance, 12 and 16 weeks following unilateral AAV delivery to the rat substantia nigra in order to establish a rat model of synuclein burden within the nigra as a model of PD in the laboratory.

3.2 Study Aims and Objectives

The aim of this study was to assess the performance of rats in a selected number of motor tasks following AAV5-CBA driven overexpression of human WT alpha-synuclein in the rat substantia nigra over a 12-16 week timeframe.

The objective of this study was to deliver AAV5-CBA-alpha-synuclein (human WT) (2µl; 1.0×10^{13} viral genomes/ml) unilaterally to the substantia nigra, and assess any contralateral performance deficits in motor tasks including the cylinder test, staircase test, open field test and amphetamine rotation test.

12 and 16 weeks post AAV delivery animals were killed humanely and their brains were processed to determine and validate expression of alpha-synuclein and perform a preliminary assessment of TH immunoreactivity as a measure of dopamine neuronal integrity within the substantia nigra.

3.3 Methods

3.3.1 Justification of Study Design

AAV5 is chosen as serotype as previous research has found it to be efficient in transducing cells in the striatum and substantia nigra (Paterna et al., 2004), as well as their availability from the Viral Vector Core of the University of Iowa on foot of funding from the Michael J Fox Foundation (MJFF). Wildtype alpha-synuclein is investigated as the accumulation of WT alpha-synuclein is associated with the development of PD in humans and it can be delivered to the rodent brain through the use of AAV vectors. The titres utilised have been validated by the MJFF and are therefore adopted in the present study. Empty vector and GFP are chosen as controls in order to control for the impact of expression of an AAV vector and a foreign protein, respectively. PBS was included as a control for the impact of injection. 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 motor behavioural tests which are carried out are the cylinder test, staircase test, open field 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 initial 12 week timepoint is chosen based on the literature reviewed above, with the findings of the 12 week study informing the decision to extend the following study to a 16 week timepoint.

3.3.2 Experimental Timeline

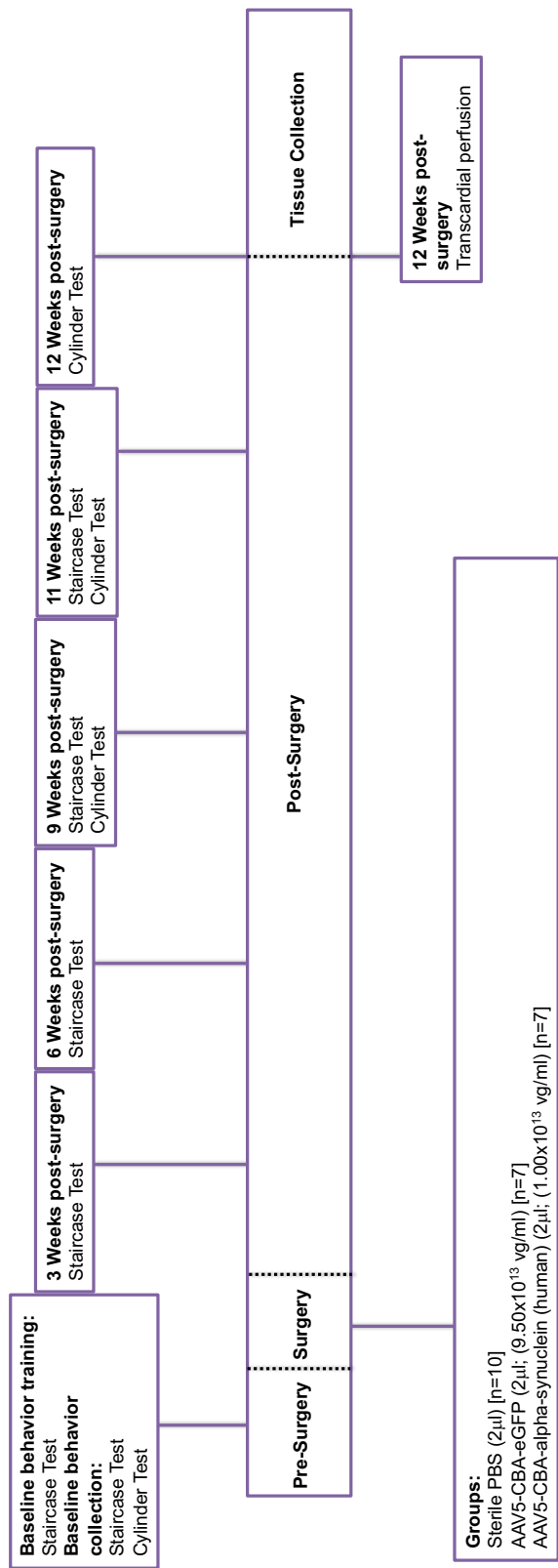


Figure 3.1: Experimental timeline for the study of unilateral AAV5-CBA human WT alpha-synuclein delivery to the rat substantia nigra on motor performance of rats over 12 weeks post-AAV delivery.

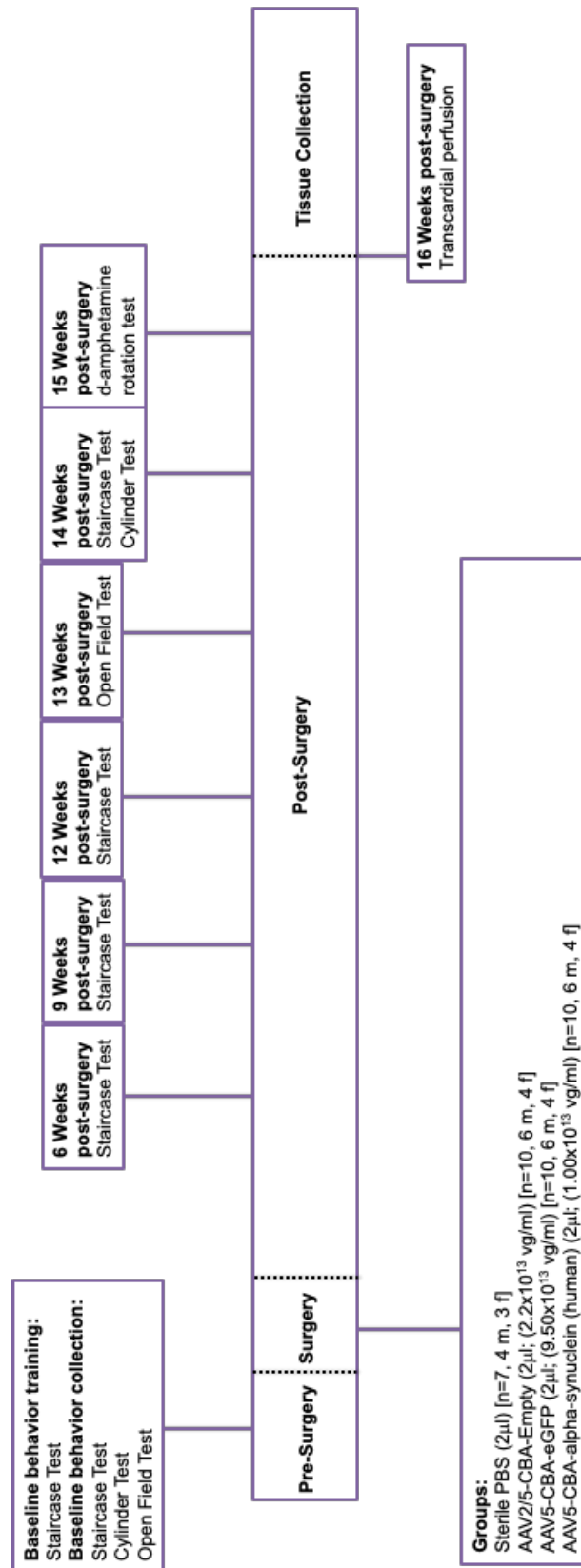


Figure 3.2: Experimental timeline for the study of unilateral AAV5-CBA human WT alpha-synuclein delivery to the rat substantia nigra on motor performance of rats over 16 weeks post-AAV delivery.

3.3.3 Experimental Design

All rats were habituated to the housing unit for at least 2 weeks before any experimental procedures commenced. During the 12 week study all rats received 5 x 10 minute training sessions in the staircase test, and in the 16 week study rats received either 3 or 4 x 10 minute training sessions, depending on the skill demonstrated when training, in order to establish proficiency in the test. Rats received a unilateral injection into the substantia nigra as outlined in Table 3.1 below.

Study	Sex	Treatment Groups Virus titre and injection volume	Coordinates
WT 12 Week Study	Male	• Sterile PBS [2µl] • AAV5-CBA-eGFP [2µl; 9.5x10¹²vg/ml] • AAV5-CBA-alpha-synuclein human WT [2µl; 1x10¹³vg/ml]	AP -5.3mm from bregma ML +2.0mm from bregma DV -8.5mm from skull
WT 16 Week Study	Male and Female	• Sterile PBS [2µl] • AAV2/5-CBA-empty [2µl; 2.2x10¹³ vg/ml] • AAV5-CBA-eGFP [2µl; 9.5x10¹²vg/ml] • AAV5-CBA-alpha-synuclein human WT [2µl; 1x10¹³vg/ml]	AP -5.3mm from bregma ML +2.0mm from bregma DV -8.0mm from skull

Table 3.1: Details of the treatment groups (Virus Titre, Injection Volumes and Coordinates) for the study of unilateral AAV5-CBA human WT alpha-synuclein delivery to the rat substantia nigra, over 12 and 16 weeks post-AAV delivery.

Behavioural testing in the staircase test, cylinder test and open field test was carried out prior to any surgical intervention as a baseline measure of performance. Post-AAV delivery behavioural testing was carried out in the staircase test, cylinder test, open field test and amphetamine rotation test, as indicated in the experimental timelines presented in Figure 3.1 and Figure 3.2.

3.3.4 Adaptations to protocols

All behavioural testing, immunohistochemistry and immunofluorescence are performed as described in Chapter 2, with the following exceptions.

3.3.4.1 *Cylinder Test*

There is an overall decrease in rearing behaviour with multiple exposures to the testing environment. It is thought that this is due to acclimatisation to the testing environment, causing the testing environment to lose its novelty and therefore lead to decreased exploration. Furthermore, it cannot be ruled out that the increase in size of the animals as the study progresses leads to a decreased exploration in the apparatus as they became too large for it (18.5cm diameter x 50 cm high). It is generally recommended that there is space between the animal and the apparatus and this was not the case as the study progressed (Schallert et al., 2000).

These factors led to the decision to adapt the testing environment at 11 and 12 weeks post-AAV delivery. The apparatus used is a black basin (50cm diameter x 39cm high). While this has the benefit of a larger testing environment which is novel to the animal, it must be noted that the cylinder is transparent and this apparatus is not, which may have additional influences on the behaviour of the animal. All behaviour is video recorded from above, which allows for scoring of wall contacts but does not allow for scoring of floor landings.

In the 16 week AAV-WT study the animals are only exposed to the testing environment twice, once to record baseline behaviour and then 14 weeks post-AAV delivery in an attempt to minimise exposure to the apparatus and associated acclimatisation and decreased exploration. All behaviour is video recorded from above, which allows for scoring of wall contacts but does not allow for scoring of floor landings.

3.3.4.2 Staircase Test

During the 12 week AAV-WT study, only the behaviour of the limb contralateral to the injection site is recorded and analysed for percentage success rate. In the 16 week AAV-WT study both limbs are recorded in order to allow the scoring of both limbs for percentage success rate.

3.3.4.3 Immunofluorescence Image Analysis

Due to the quality of the immunofluorescence the below protocol is adopted to analyse the immunofluorescence intensity. 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. This is achieved by starting the threshold where the histogram begins to flatten out, to the right of the peak of the histogram where most of the background noise is contained. The Analyse -> Measure function is then utilised on Image J. From this the mean pixels value/arbitrary units value is generated. This is then expressed as a percentage of the un-injected side, as well as being expressed as arbitrary units.

3.4 Results – 12 Week AAV-WT Study

3.4.1 Alpha-synuclein Immunofluorescence

3.4.1.1 Increased alpha-synuclein immunofluorescence was identified in the substantia nigra of the injected hemisphere in comparison to the un-injected hemisphere when immunofluorescence levels of alpha-synuclein were assessed 12 weeks post-AAV delivery.

Comparison of the injected and un-injected hemispheres of the substantia nigra in the AAV-WT AAV-alpha-synuclein injected group found increased intensity of the alpha-synuclein immunofluorescence in the injected hemisphere when compared to the un-injected hemisphere ($t(5) = 2.779$, $p = 0.0389$) (Figure 3.3).

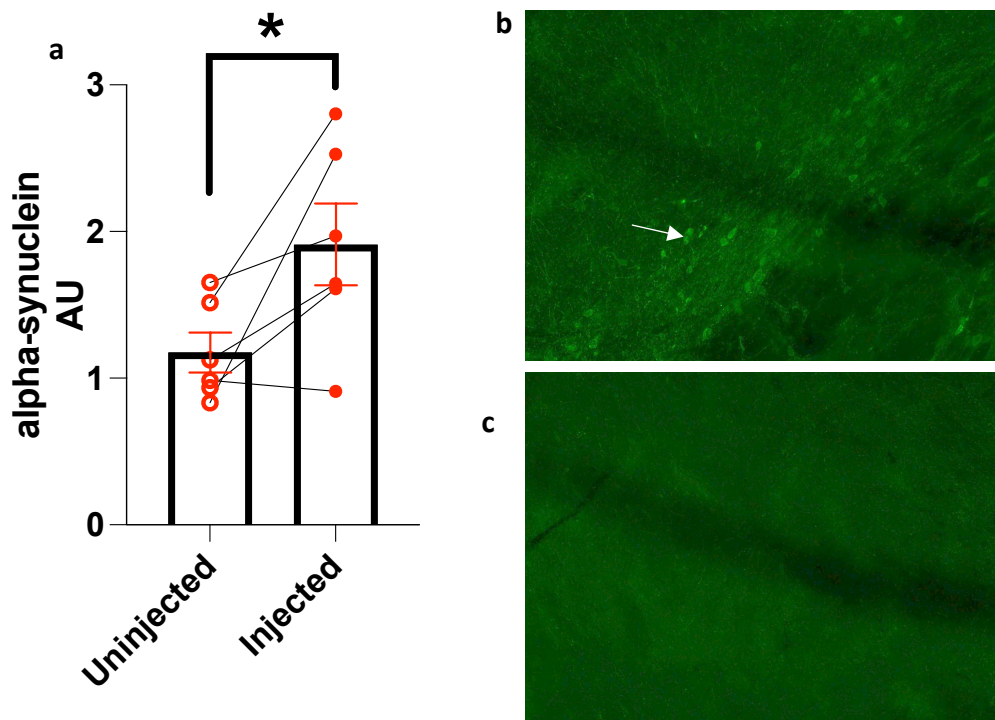


Figure 3.3: Alpha-synuclein immunofluorescence in the substantia nigra of the un-injected and injected hemispheres of the AAV-WT alpha-synuclein group at 12 weeks post-AAV delivery. (a) Increased alpha-synuclein immunofluorescence was identified in the injected hemisphere when compared with the un-injected hemisphere. (b) Representative image from the injected hemisphere. (c) Representative image from the un-injected hemisphere. Data presented as mean with standard error of the mean (SEM). Paired t-test. $n = 6$. Images acquired at 20x magnification. * $p < 0.05$. AU = arbitrary units. Arrow indicating alpha-synuclein immunofluorescence.

3.4.2 GFP Immunofluorescence

3.4.2.1 An increase in GFP immunofluorescence was identified in the substantia nigra of the injected hemisphere when compared to the un-injected hemisphere when immunofluorescence levels were assessed at 12 weeks post-AAV delivery.

Comparison of the injected and un-injected hemispheres of the substantia nigra in the AAV-eGFP injected group found a difference between GFP immunofluorescence in the injected compared to the un-injected hemisphere ($t(5) = 2.958$, $p = 0.0316$) (Figure 3.4).

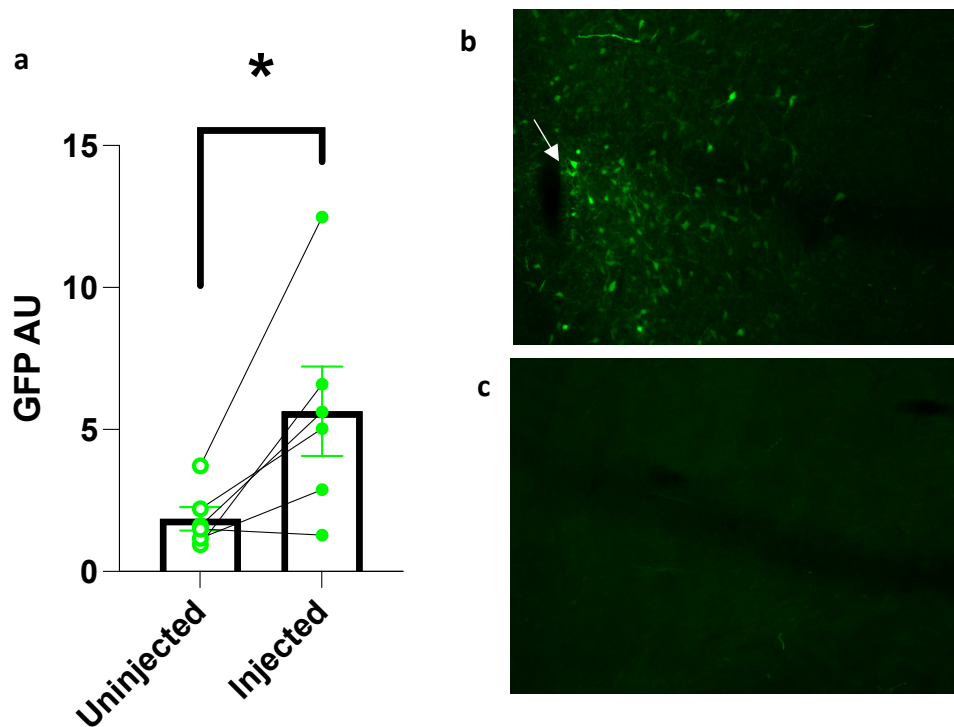


Figure 3.4: GFP immunofluorescence in the substantia nigra of the un-injected and injected hemispheres of the AAV-eGFP group at 12 weeks post-AAV delivery. (a) Increased GFP immunofluorescence was identified in the injected hemisphere when compared with the un-injected hemisphere. (b) Representative image of the injected hemisphere. (c) Representative image of the un-injected hemisphere. Data presented as mean with SEM. Paired t-test. $n = 6$. Image acquired at 20x magnification. * $p < 0.05$. AU = arbitrary units. Arrow indicating GFP immunofluorescence.

3.4.3 Staircase Test

3.4.3.1 Intranigral injection of AAV5-CBA-alpha-synuclein did not lead to the development of motor deficits in the staircase test across multiple time-points post-AAV delivery.

Three animals in the PBS group and two animals in the GFP group were removed from analysis as they met the criteria for unsuccessful acquisition of the staircase test as outlined in Table 2.2. No effects were identified for percentage success at baseline ($F(2, 16) = 0.0079, p = 0.9922$). Similarly analysis of the number of pellets retrieved found no effects (Interaction $F(2, 32) = 0.3941, p = 0.6775$; Side $F(1, 32) = 0.0239, p = 0.8782$; Treatment $F(2, 32) = 0.2616, p = 0.7715$).

At three weeks post-AAV delivery no effects were identified for percentage success ($F(2, 16) = 1.963, p = 0.1728$). Similarly analysis of the number of pellets retrieved found no effects (Interaction $F(2, 32) = 0.3519, p = 0.7061$; Side $F(1, 32) = 0.4913, p = 0.4884$; Treatment $F(2, 32) = 2.508, p = 0.0973$).

The staircase test was repeated at 6 weeks post-AAV delivery and no effects were identified for percentage success ($F(2, 16) = 1.918, p = 0.1792$) or number of pellets retrieved (Interaction $F(2, 32) = 0.4139, p = 0.6645$; Side $F(1, 32) = 0.0104, p = 0.9196$; Treatment $F(2, 32) = 0.5689, p = 0.5718$).

At nine weeks post-AAV delivery no effects were identified for percentage success ($F(2, 16) = 2.074, p = 0.1581$) or number of pellets retrieved (Interaction $F(2, 32) = 0.2249, p = 0.7998$; Side $F(1, 32) = 0.3578, p = 0.5539$; Treatment $F(2, 32) = 0.5998, p = 0.5550$).

Finally, the staircase test was repeated at 11 weeks post-AAV delivery and no effects were identified when percentage success was analysed ($F(2, 16) = 2.074, p = 0.1581$). Similarly, no effects were identified when number of pellets retrieved was analysed (Interaction $F(2, 32) = 0.0995, p = 0.9055$; Side $F(1, 32) = 0.0927, p = 0.7627$; Treatment $F(2, 32) = 2.023, p = 0.1448$) (Figure 3.5).

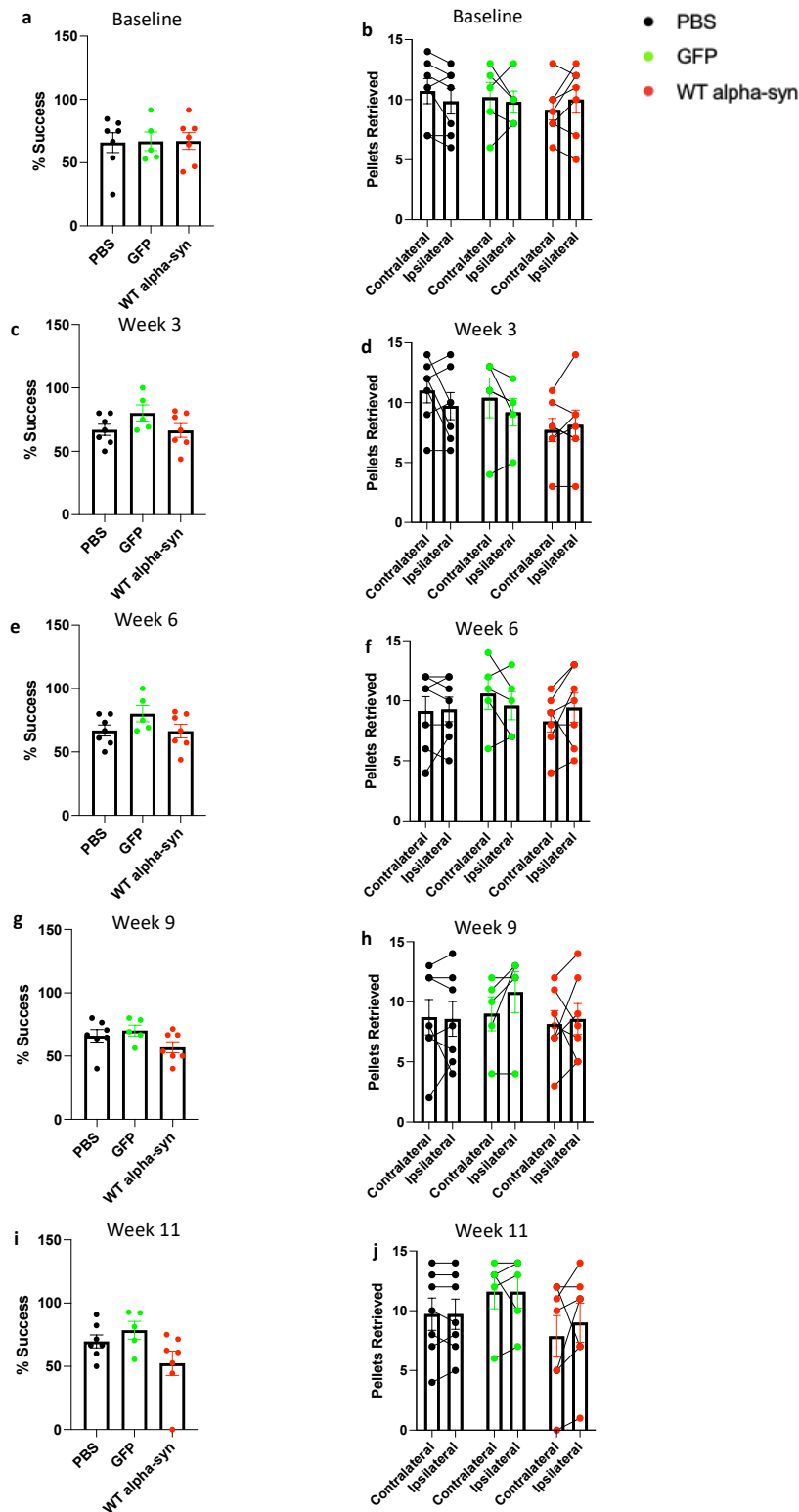


Figure 3.5: Percentage success and number of pellets retrieved in the staircase test prior to AAV-delivery and at multiple time points post-AAV delivery. No differences were identified between the treatment groups at baseline (a and b), 3 weeks post-AAV delivery (c and d), 6 weeks post-AAV delivery (e and f), 9 weeks post-AAV delivery (g and h), or 11 weeks post-AAV delivery (i and j). Data presented as mean with SEM. One-way ANOVA (a, c, e, g, i) or two-way ANOVA (b, d, f, h, j). $n = 5-7$.

3.4.4 Cylinder Test

3.4.4.1 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 ipsilateral wall contacts ($F(2, 20) = 0.1482, p = 0.8632$), percentage ipsilateral 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 ($H(2) = 0.3859, p = 0.8245$). Data presented in Appendix I.

3.4.4.2 Intranigral injection of AAV5-CBA-alpha-synuclein did not lead to the development of limb use asymmetry at nine weeks post-AAV delivery.

No effects were identified at nine weeks post-AAV delivery for number of contralateral wall contacts ($H(2) = 4.583, p = 0.1011$), percentage contralateral wall contacts ($F(2, 21) = 0.6375, p = 0.5386$), number of ipsilateral wall contacts ($F(2, 21) = 0.0379, p = 0.9628$), percentage ipsilateral wall contacts ($H(2) = 1.373, p = 0.5033$), number of simultaneous wall contacts ($H(2) = 2.794, p = 0.2473$), or percentage simultaneous wall contacts ($F(2, 21) = 1.279, p = 0.2993$) (Figure 3.6).

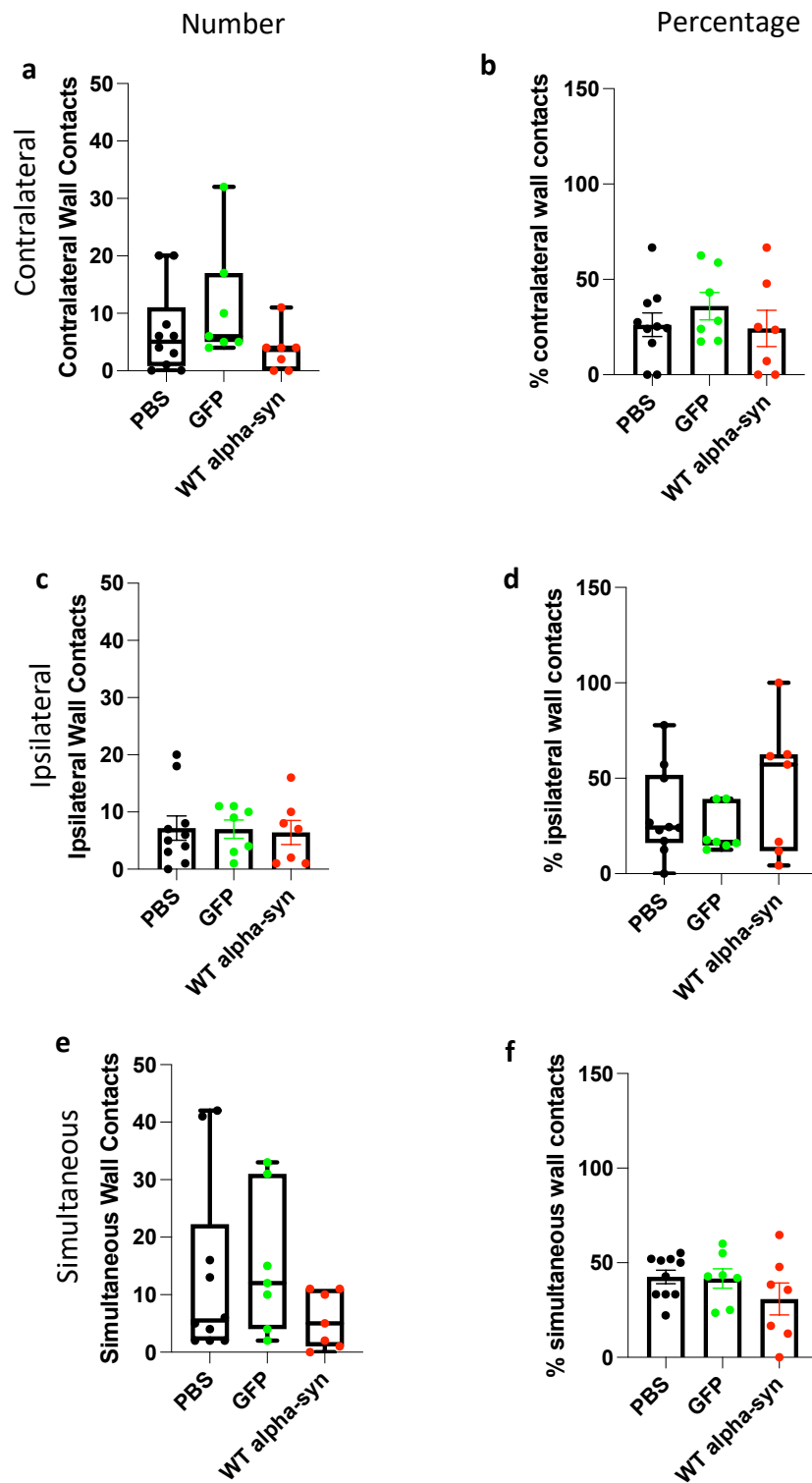


Figure 3.6: Performance in the cylinder test at nine weeks post-AAV delivery. No differences were identified between the treatment groups at nine weeks post-AAV delivery. Data presented as mean with SEM (b, c, f) or median with minimum to maximum (a, d, e). One-way ANOVA (b, c, f) or Kruskal-Wallis test (b, c, f). n = 7-10.

3.4.4.3 The majority of rearing behaviour occurs during the first minute of exposure to the cylinder apparatus.

Decreased total number of rears was observed when the rearing behaviour for the animals across all three treatment groups was assessed pre-AAV delivery (Interaction $F(8, 84) = 1.879$, $p = 0.0740$; Time $F(3.100, 65.11) = 65.78$, $p < 0.0001$; Treatment $F(2, 21) = 0.6878$; $p = 0.5137$), with post-hoc comparisons showing that minutes 2, 3, 4, and 5 had less rears than minute 1 ($p < 0.0001$). A similar effect was observed at 9 weeks post-AAV delivery (Interaction $F(8, 84) = 1.163$, $p = 0.3313$; Time $F(3.255, 68.35) = 3.992$, $p = 0.0093$; Treatment $F(2, 21) = 0.8208$, $p = 0.4537$), with post-hoc comparisons showing that minute 5 had less rears than minute 1 ($p < 0.05$) (Figure 3.7).

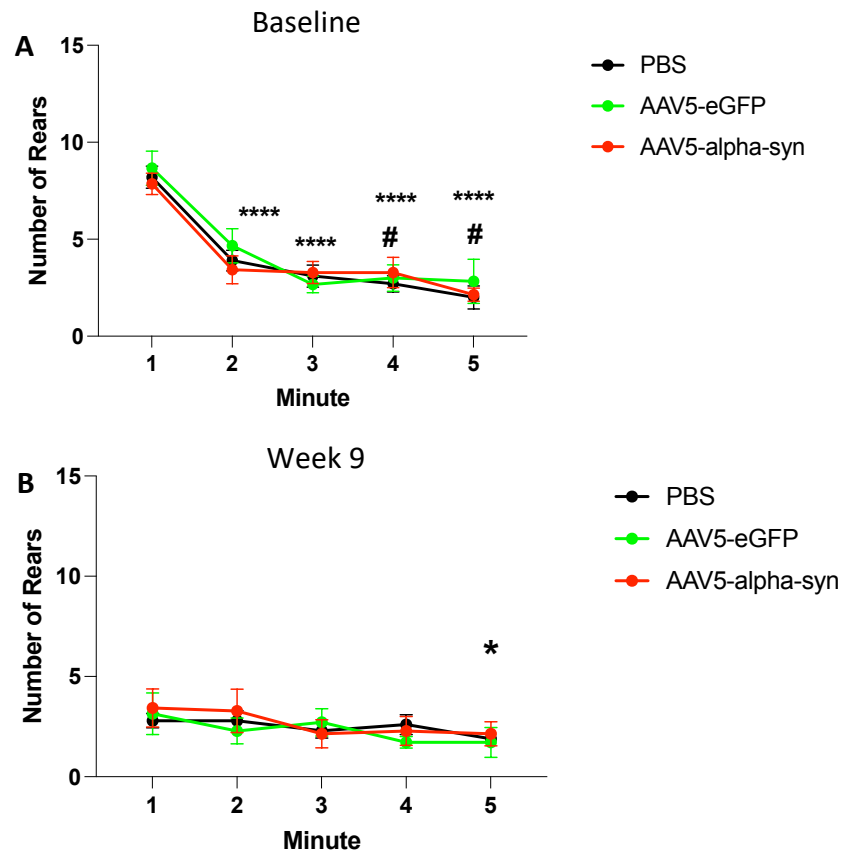


Figure 3.7: Decreased rears were identified over the 5 minute testing period pre-AAV delivery and at nine weeks post-AAV delivery. An effect of time was found at both baseline (A) and nine weeks post-AAV delivery (B). Data expressed as mean with SEM. Two-way repeated measures ANOVA followed by Tukey's multiple comparisons test. $n = 7-10$ per group. * $p < 0.05$, **** $p < 0.0001$ vs minute 1; # $p < 0.05$ vs minute 2.

3.4.4.4 Intranigral injection of AAV5-CBA-alpha-synuclein led to a tendency towards decreased contralateral limb contacts in the WT alpha-synuclein group at 11 weeks post-AAV delivery in a modified cylinder test.

An effect was identified for the number of contralateral wall contacts at 11 weeks post-AAV delivery ($H(2) = 7.273, p = 0.0263$). Post-hoc analysis did not identify any differences between the groups, however a trend towards a significant difference was identified between the PBS and AAV-WT alpha-synuclein groups ($p = 0.0554$), and between the AAV-eGFP and AAV-WT alpha-synuclein groups ($p = 0.0519$). No effects were identified at 11 weeks post-AAV delivery for percentage contralateral wall contacts ($F(2, 21) = 1.764, p = 0.1958$), number of ipsilateral wall contacts ($F(2.000, 13.94) = 1.235, p = 0.3208$), percentage ipsilateral wall contacts ($F(2, 21) = 3.312, p = 0.0562$), number of simultaneous wall contacts ($F(2, 21) = 1.249, p = 0.3072$), or percentage simultaneous wall contacts ($F(2, 21) = 1.105, p = 0.3497$) (Figure 3.8).

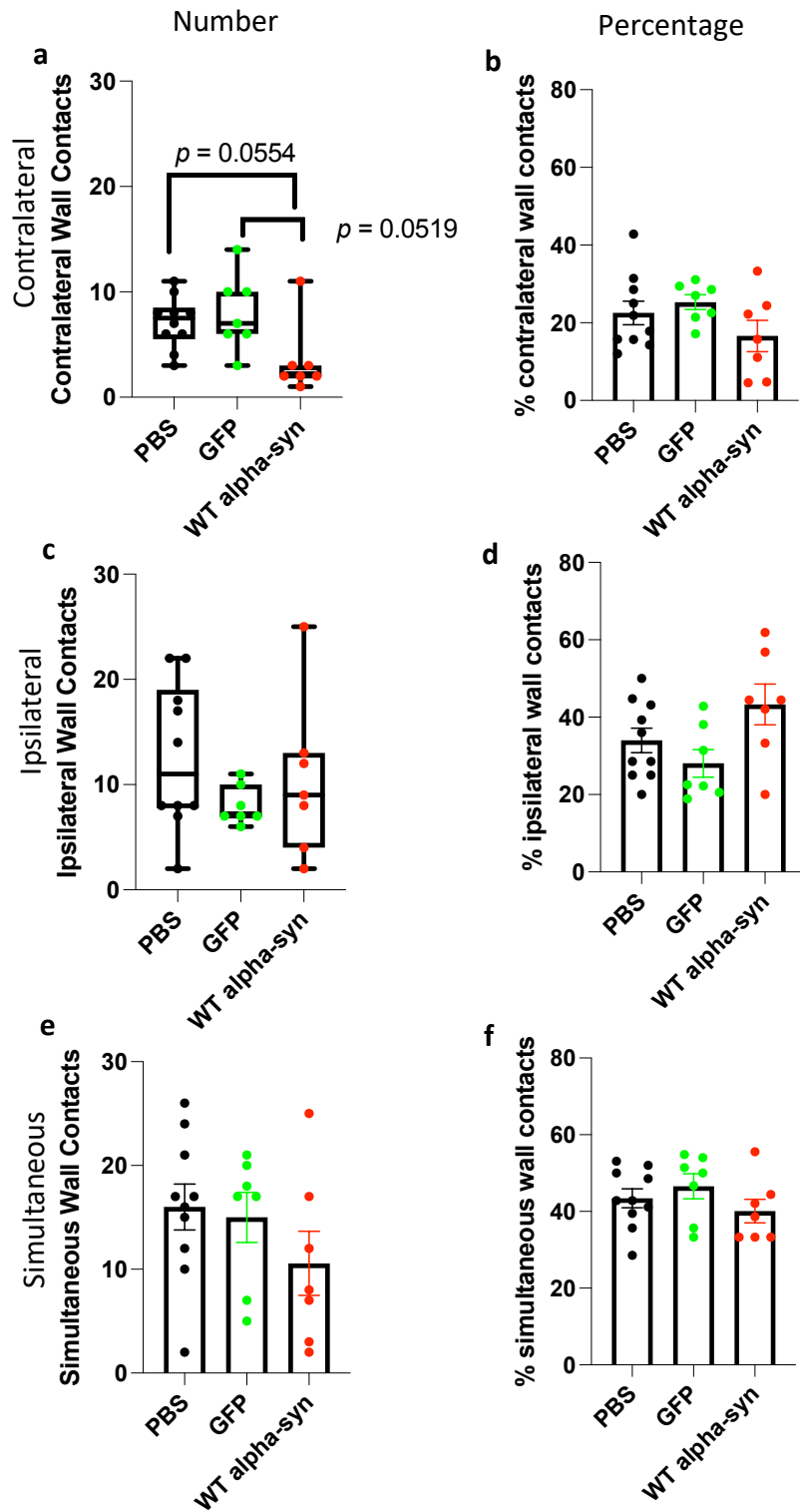


Figure 3.8: Performance in the cylinder test at 11 weeks post-AAV delivery. Analysis of performance in the cylinder test at 11 weeks post-AAV delivery found no differences between the treatment groups, however the AAV-WT alpha-synuclein group showed a tendency towards decreased contralateral wall contacts when compared with AAV-eGFP and PBS (a). Data presented as mean with SEM (b, d, e, f) or median with interquartile range (a, c). One-way ANOVA (b, d, e, f), Brown-Forsythe ANOVA (c) or Kruskal-Wallis (a) test followed by Dunn's multiple comparisons test. $n = 7-10$.

3.4.4.5 Intranigral injection of AAV5-CBA-alpha-synuclein did not lead to the development of limb use asymmetry at 12 weeks post-AAV delivery in a modified cylinder test.

No effects were identified between the groups at 12 weeks post-AAV delivery for number of contralateral wall contacts ($H(2) = 1.678, p = 0.4321$), percentage contralateral wall contacts ($H(2) = 1.803, p = 0.4060$), number of ipsilateral wall contacts ($F(2, 21) = 0.2064, p = 0.8151$), percentage ipsilateral wall contacts ($F(2, 21) = 1.077, p = 0.3586$), number of simultaneous wall contacts ($H(2) = 1.077, p = 0.5835$), or percentage simultaneous wall contacts ($F(2, 21) = 1.377, p = 0.2741$) (Figure 3.9).

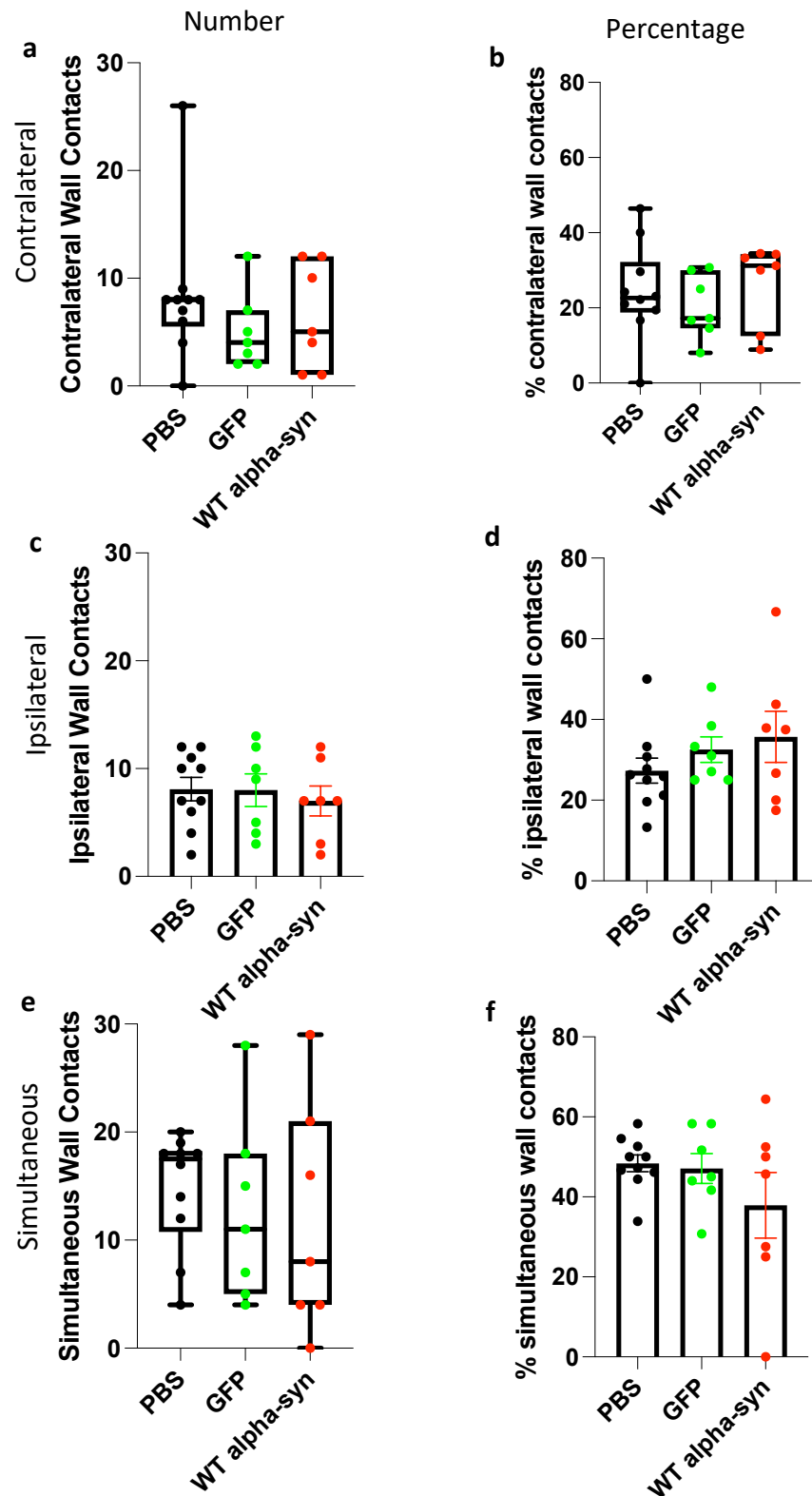


Figure 3.9: Performance in the cylinder test at 12 weeks post-AAV delivery. No differences were identified between the treatment groups at 12 weeks post-AAV delivery. Data presented as mean with SEM (c, d, f) or median with minimum and maximum (a, b, e). One-way ANOVA (c, d, f) or Kruskal-Wallis test (a, b, e). n = 7-10.

3.4.4.6 Intraneural injection of AAV5-CBA- α -synuclein did not lead to the development of limb use asymmetry as assessed by the limb use asymmetry score.

Limb use asymmetry was assessed pre-AAV delivery, and again at nine, 11 and 12 weeks post-AAV delivery. No effects were identified pre-AAV delivery ($F(2, 20) = 0.0851, p = 0.9187$), nine weeks post-AAV delivery ($F(2, 21) = 0.2823, p = 0.7569$), 11 weeks post-AAV delivery ($F(2, 21) = 3.019, p = 0.0704$) or 12 weeks post-AAV delivery ($F(2, 21) = 0.5411, p = 0.5900$) (Figure 3.10).

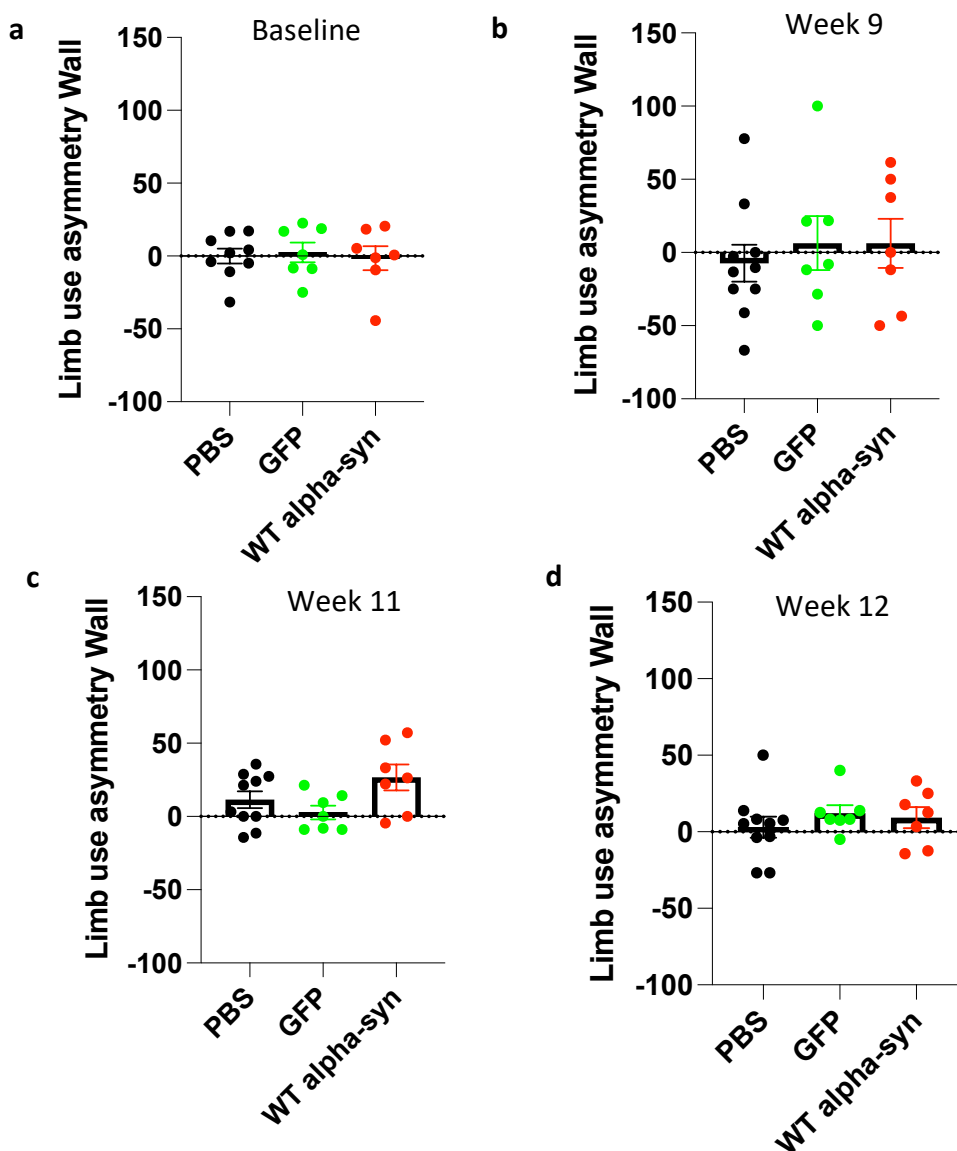


Figure 3.10: Limb use asymmetry across multiple timepoints in the cylinder test. Analysis of limb use asymmetry found no effects pre-AAV delivery (a), 9 weeks post-AAV delivery (b), 11 weeks post-AAV delivery (c), or 12 weeks post-AAV delivery (d). Data presented as mean with SEM. One-way ANOVA. $n = 7-10$.

3.4.5 Tyrosine Hydroxylase Immunoreactive Cells

3.4.5.1 Tyrosine hydroxylase (TH) immunoreactive cells following AAV-alpha-synuclein delivery 12 weeks post AAV-delivery to the rat substantia nigra.

Tyrosine hydroxylase immunoreactive cell quantification was not possible in this study as the injected hemisphere was not marked during brain sectioning and therefore could not be identified when using 3, 3'-diaminobenzidine (DAB) immunohistochemistry. While immunofluorescence was performed for both alpha-synuclein and tyrosine hydroxylase, tyrosine hydroxylase cell counting through immunofluorescence was not performed due to technical limitations.

3.4.6 Summary of results – 12 Week AAV-WT Study

Staircase Test (multiple timepoints)	Cylinder Test (multiple timepoints)	TH immunoreactivity
No differences	Trend towards decreased number of contralateral wall contacts in both the AAV-WT-alpha-synuclein and AAV-eGFP groups at 11 weeks post-AAV delivery in a modified version of cylinder test.	Not assessed

Table 3.2: A summary of the results obtained in the 12 week AAV-WT study.

3.5 Results – 16 week AAV-WT Study

3.5.1 Alpha-synuclein Immunofluorescence

3.5.1.1 Increased alpha-synuclein immunofluorescence was identified in the injected hemisphere in comparison to the un-injected hemisphere when immunofluorescence levels of alpha-synuclein were assessed 16 weeks post-AAV delivery.

Comparison of the injected and un-injected hemispheres in the AAV-WT alpha-synuclein injected group found increased intensity of alpha-synuclein immunofluorescence in the injected hemisphere when compared to the un-injected hemisphere ($t(9) = 6.417$, $p = 0.0001$) (Figure 3.11).

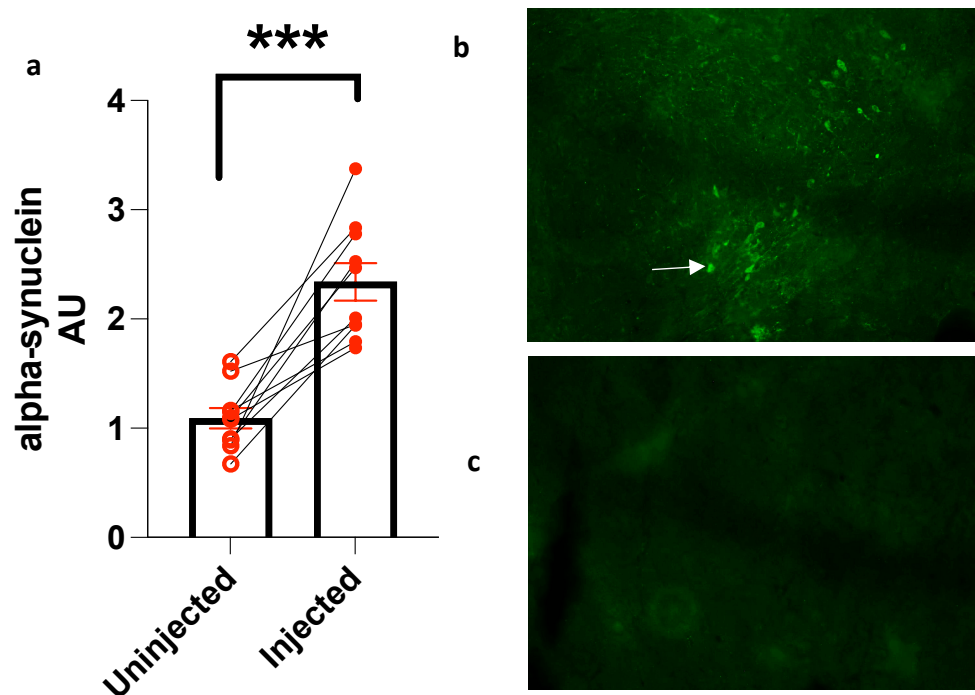


Figure 3.11: Alpha-synuclein immunofluorescence in the substantia nigra of the un-injected and injected hemispheres of the AAV-WT alpha-synuclein group at 16 weeks post-AAV delivery with both sexes combined. (a) Increased alpha-synuclein immunofluorescence was identified in the injected hemisphere when compared with the un-injected hemisphere. (b) Representative image from the injected hemisphere. (c) Representative image from the un-injected hemisphere. Data presented as mean with SEM. Paired t-test. $n = 10$. Images acquired at 20x magnification. *** $p < 0.001$. AU = arbitrary units. Arrow indicating positive alpha-synuclein immunofluorescence.

3.5.1.2 Increased alpha-synuclein immunofluorescence was identified in the injected hemisphere in comparison to the un-injected hemisphere when immunofluorescence levels of alpha-synuclein were assessed 16 weeks post-AAV delivery for males and females separately.

Comparison of the injected and un-injected hemispheres in the AAV-WT alpha-synuclein injected group found increased intensity of the alpha-synuclein immunofluorescence in the injected hemisphere when compared to the un-injected hemisphere in males ($t(5) = 5.452$, $p = 0.0028$) and in females ($t(3) = 3.604$, $p = 0.0366$) (Figure 3.12).

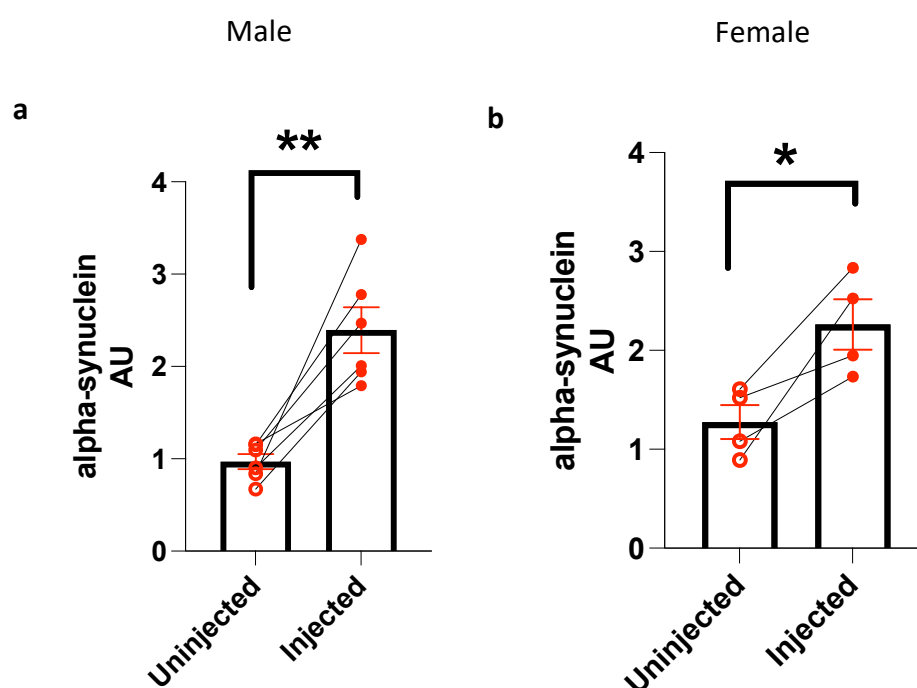


Figure 3.12: Alpha-synuclein immunofluorescence in the substantia nigra of the un-injected and injected hemispheres of the AAV-WT alpha-synuclein group at 16 weeks post-AAV delivery for males (a) and females (b) separately. Increased alpha-synuclein immunofluorescence was identified in the injected hemisphere when compared with the un-injected hemisphere when males were analysed alone (a) and when females were analysed alone (b). Data presented as mean with SEM. Paired t-test. $n = 4-6$. * $p < 0.05$, ** $p < 0.01$. AU = arbitrary units.

3.5.2 Staircase Test

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

Two animals in the Empty group, five animals in the GFP group, and four animals in the WT alpha-syn group were removed from analysis as they met the criteria for unsuccessful acquisition of the staircase test as outlined in Table 2.2. 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. Data presented in Appendix I.

3.5.2.2 No differences were identified between the groups in the staircase test at six weeks post-AAV delivery.

Testing at six weeks post-AAV delivery found no effects for number of pellets retrieved with both sexes combined (Interaction $F(3, 44) = 0.5319$, $p = 0.6628$; Side $F(1, 44) = 1.207$, $p = 0.2779$; Treatment $F(3, 44) = 0.3107$, $p = 0.8176$) or for males alone (Interaction $F(3, 22) = 0.1882$, $p = 0.9033$; Side $F(1, 22) = 0.0896$, $p = 0.7675$; Treatment $F(3, 22) = 1.245$, $p = 0.3175$).

Analysis of percentage success at six weeks post-AAV delivery found an effect of side when males were analysed alone (Interaction $F(3, 22) = 1.354$, $p = 0.2828$; Side $F(1, 22) = 5.183$, $p = 0.0329$; Treatment $F(3, 22) = 0.3795$, $p =$

0.7687). Post-hoc comparisons did not identify any differences between the treatment groups.

No effects were identified for percentage success when both were sexes combined (Interaction $F(3, 44) = 0.3207$, $p = 0.8103$; Side $F(1, 44) = 2.103$, $p = 0.1541$; Treatment $F(3, 44) = 0.4981$, $p = 0.6855$). Due to the low number of females statistical analysis was not performed for females alone, with the data graphically presented in Figure 3.13.

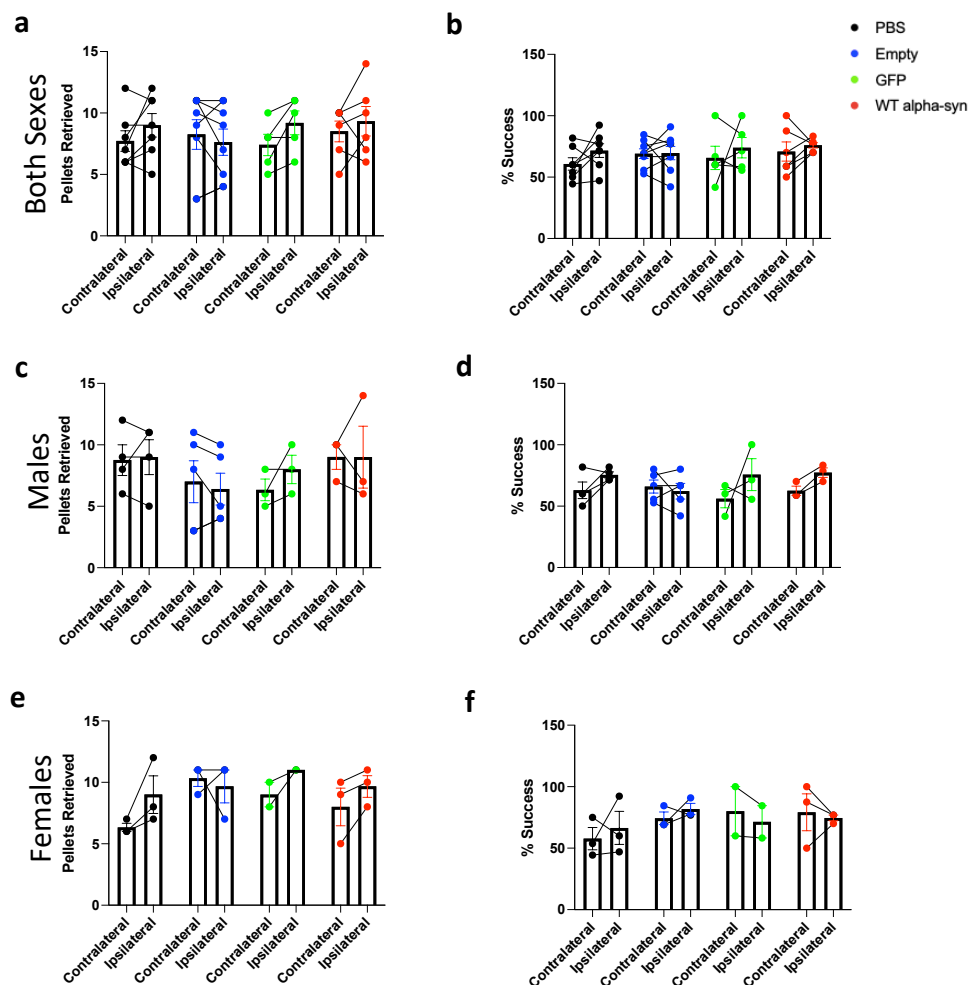


Figure 3.13: Number of pellets retrieved and percentage success at six weeks post-AAV delivery in the staircase test. No differences between the treatment groups were identified at six weeks post-AAV delivery. Data presented as mean with SEM. Two-way ANOVA. $n = 5 - 8$ (a, b) both sexes, $n = 3 - 5$ (c, d) males, $n = 2 - 3$ (e, f) females.

3.5.2.3 No differences were identified between the groups in the staircase test at nine weeks post-AAV delivery.

Testing at nine weeks post-AAV delivery found an effect of side for number of pellets retrieved with both sexes combined (Interaction $F(3, 44) = 0.051$, $p = 0.9846$; Side $F(1, 44) = 6.553$, $p = 0.014$; Treatment $F(3, 44) = 0.4190$, $p = 0.7403$). Post-hoc comparisons identified no differences between the treatment groups.

Analysis of males alone also identified an effect of side (Interaction $F(3, 22) = 0.3583$, $p = 0.7837$; Side $F(1, 22) = 10.91$, $p = 0.0032$; Treatment $F(3, 22) = 2.092$, $p = 0.1304$). Post-hoc comparisons identified no differences between the treatment groups.

Analysis of the success rate at nine weeks post-AAV delivery found no effects with both sexes combined (Interaction $F(3, 44) = 1.339$, $p = 0.2739$; Side $F(1, 44) = 0.0024$, $p = 0.9615$; Treatment $F(3, 44) = 0.8938$, $p = 0.4519$) or when males were analysed alone (Interaction $F(3, 22) = 1.179$, $p = 0.3403$; Side $F(1, 22) = 1.457$, $p = 0.2402$; Treatment $F(3, 22) = 1.528$, $p = 0.2351$). Due to the low number of females statistical analysis was not performed for females alone, with the data graphically presented in Figure 3.14.

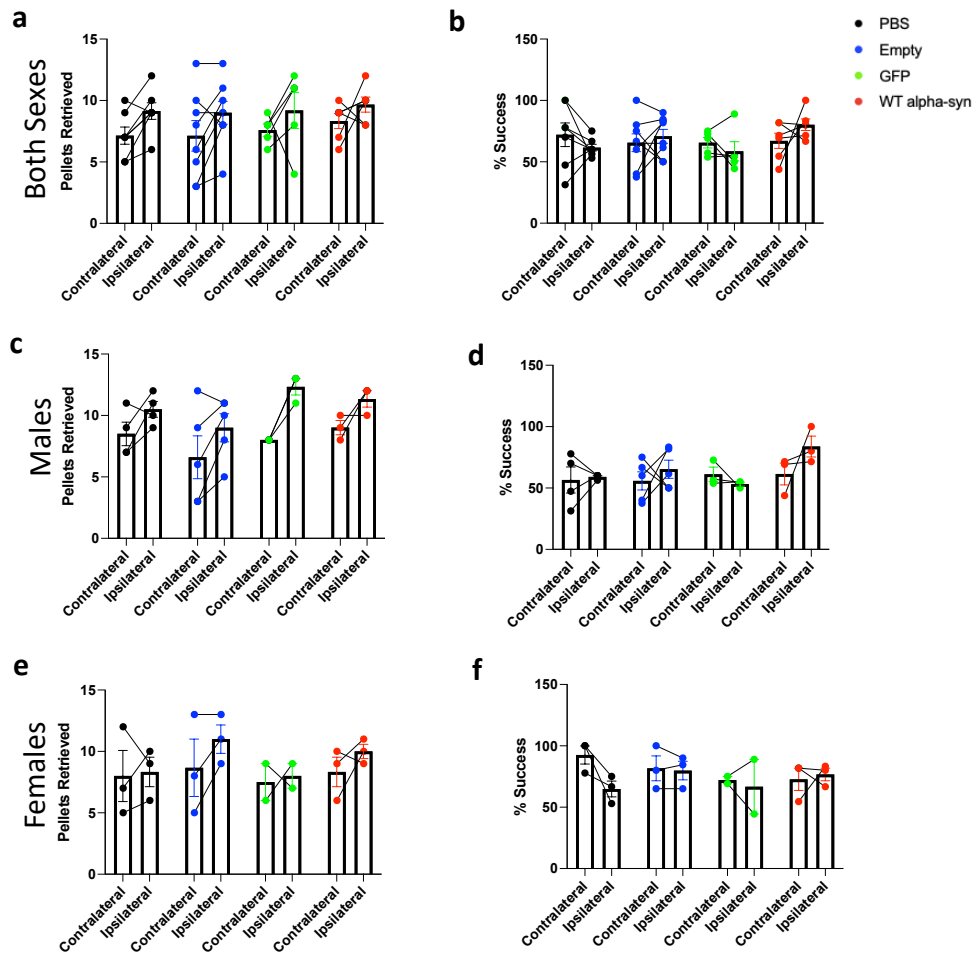


Figure 3.14: Number of pellets retrieved and percentage success at nine weeks post-AAV delivery in the staircase test. No differences between the treatment groups were identified at nine weeks post-AAV delivery. Data presented as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. $n = 5 - 8$ (a, b) both sexes combined, $n = 3 - 5$ (c, d) males, $n = 2 - 3$ (e, f) females.

3.5.2.4 No differences were identified between the groups in the staircase test at 12 weeks post-AAV delivery.

Testing at 12 weeks post-AAV delivery found no effects for number of pellets retrieved when both sexes were analysed together (Interaction $F(3, 44) = 0.1363$, $p = 0.9378$; Side $F(1, 44) = 0.6232$, $p = 0.4341$; Interaction $F(3, 44) = 1.736$, $p = 0.1735$).

Analysis of males alone identified an effect of treatment for number of pellets retrieved (Interaction $F(3, 22) = 0.0391$, $p = 0.9894$; Side $F(1, 22) = 0.1288$, $p = 0.7231$; Treatment $F(3, 22) = 3.062$, $p = 0.0494$). Post-hoc comparisons did not identify any differences between the treatment groups.

Analysis of percentage success at 12 weeks post-AAV delivery identified no effects when both sexes were analysed in combination (Interaction $F(3, 44) = 0.4643$, $p = 0.7086$; Side $F(1, 44) = 0.1643$, $p = 0.6872$; Treatment $F(3, 44) = 2.317$, $p = 0.0887$).

Analysis of males alone identified an effect of treatment (Interaction $F(3, 22) = 0.1388$, $p = 0.9357$; Side $F(1, 22) = 0.8348$, $p = 0.3708$; Treatment $F(3, 22) = 4.504$, $p = 0.0131$). Post-hoc comparisons did not identify any differences between the treatment groups. Due to the low number of females statistical analysis was not performed for females alone, with the data graphically presented in Figure 3.15.

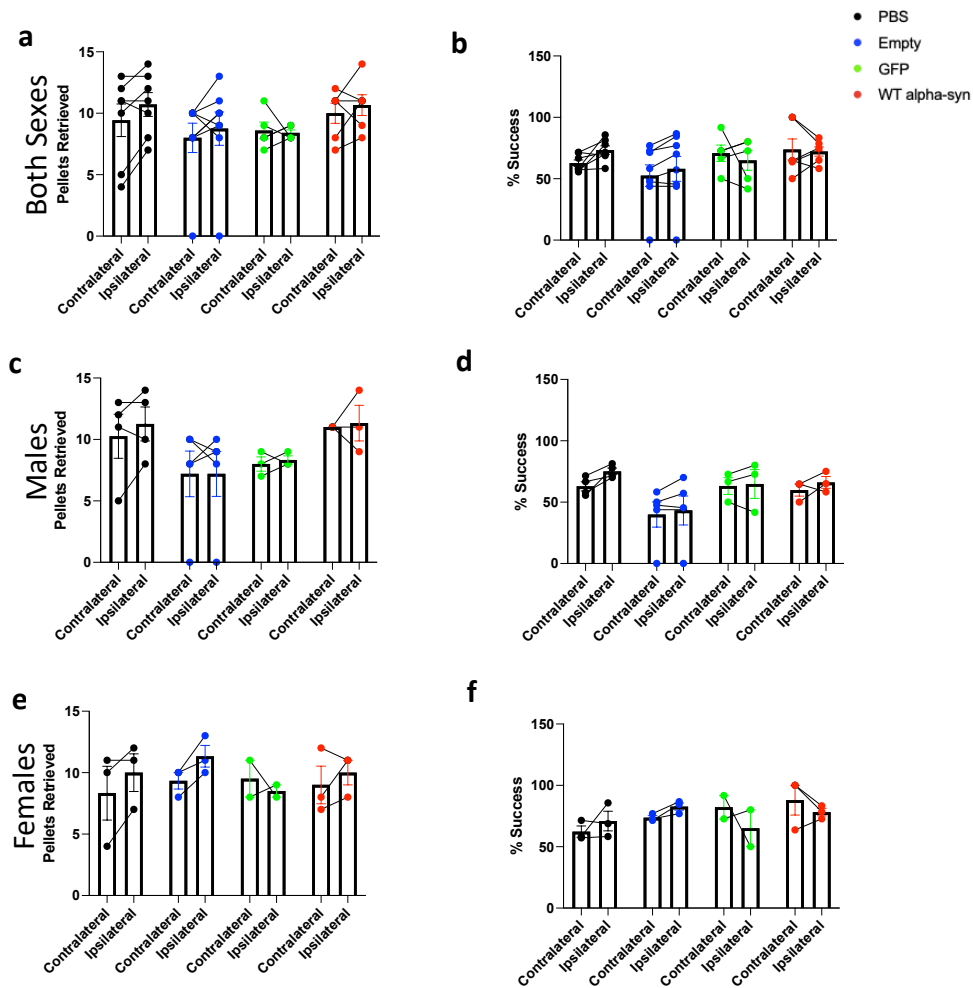


Figure 3.15: Number of pellets retrieved and percentage success at 12 weeks post-AAV delivery in the staircase test. No differences between the treatment groups were identified at 12 weeks post-AAV delivery. Data presented as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 5 – 8 (a, b) both sexes combined, n = 3 – 5 (c, d) males, n = 2 – 3 (e, f) females.

3.5.2.5 No differences were identified between the groups in the staircase test at 14 weeks post-AAV delivery.

Testing at fourteen weeks post-AAV delivery found no effects for number of pellets retrieved when both sexes were analysed together (Interaction $F(3, 44) = 0.2204$, $p = 0.8817$; Side $F(1, 44) = 2.237$, $p = 0.1419$; Treatment $F(3, 44) = 0.7932$, $p = 0.5042$) or when males were analysed alone (Interaction $F(3, 22) = 0.0447$, $p = 0.9871$; Side $F(1, 22) = 2.525$, $p = 0.1263$; Treatment $F(3, 22) = 1.015$, $p = 0.4050$).

Analysis of success rate at fourteen weeks post-AAV delivery did not identify any effects when both sexes were analysed together (Interaction $F(3, 44) = 2.393$, $p = 0.0812$; Side $F(1, 44) = 1.324$, $p = 0.2560$; Treatment $F(3, 44) = 0.1142$, $p = 0.9514$).

Analysis of males alone identified an interaction effect and an effect of side (Interaction $F(3, 22) = 3.288$, $p = 0.0397$; Side $F(1, 22) = 5.430$, $p = 0.0294$; Treatment $F(3, 22) = 0.9887$, $p = 0.4163$), with post-hoc comparisons failing to identify any differences between the treatment groups. Due to the low number of females statistical analysis was not performed for females alone, with the data graphically presented in Figure 3.16.

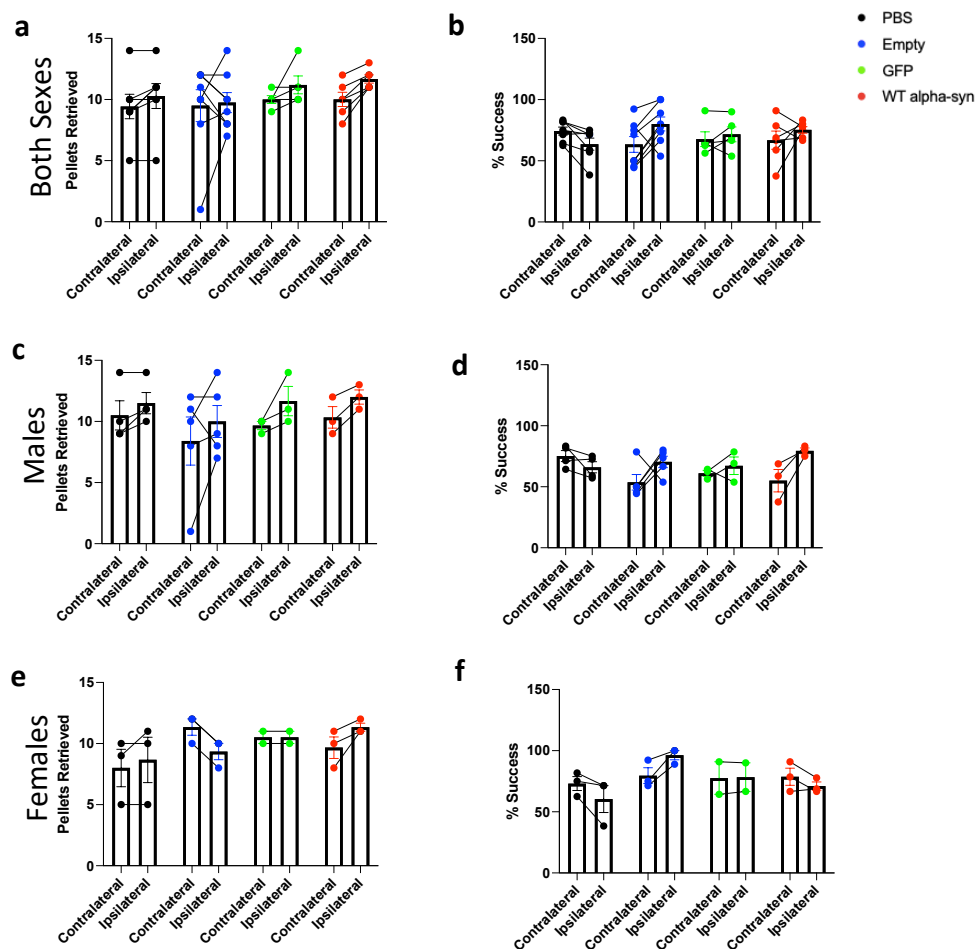


Figure 3.16: Number of pellets retrieved and percentage success at 14 weeks post-AAV delivery in the staircase test. No differences between the treatment groups were identified at 14 weeks post-AAV delivery. Data presented as mean with SEM. Two-way ANOVA followed by Newman-Keuls multiple comparisons test. n = 5 – 8 (a, b) both sexes combined, n = 3 – 5 (c, d) males, n = 2 – 3 (e, f) females.

3.5.3 Cylinder Test

3.5.3.1 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 ($F(3, 30) = 0.1183, p = 0.9496$). Similarly, no differences were identified when males or females were analysed separately. Data presented in Appendix I.

3.5.3.2 Intranigral injection of AAV5-CBA-alpha-synuclein did not lead to the development of limb use asymmetry at 14 weeks post-AAV delivery.

No effects were identified at 14 weeks post-AAV delivery for number of contralateral wall contacts ($H(3) = 0.6009, p = 0.8962$), percentage contralateral wall contacts ($F(3, 32) = 0.6632, p = 0.5808$), number of ipsilateral wall contacts ($F(3, 32) = 0.1706, p = 0.9155$), percentage ipsilateral wall contacts ($H(3) = 3.261, p = 0.3530$), number of simultaneous wall contacts ($F(3, 32) = 0.3665, p = 0.7776$), or percentage simultaneous wall contacts ($F(3, 32) = 0.3813, p = 0.7671$) (Figure 3.17).

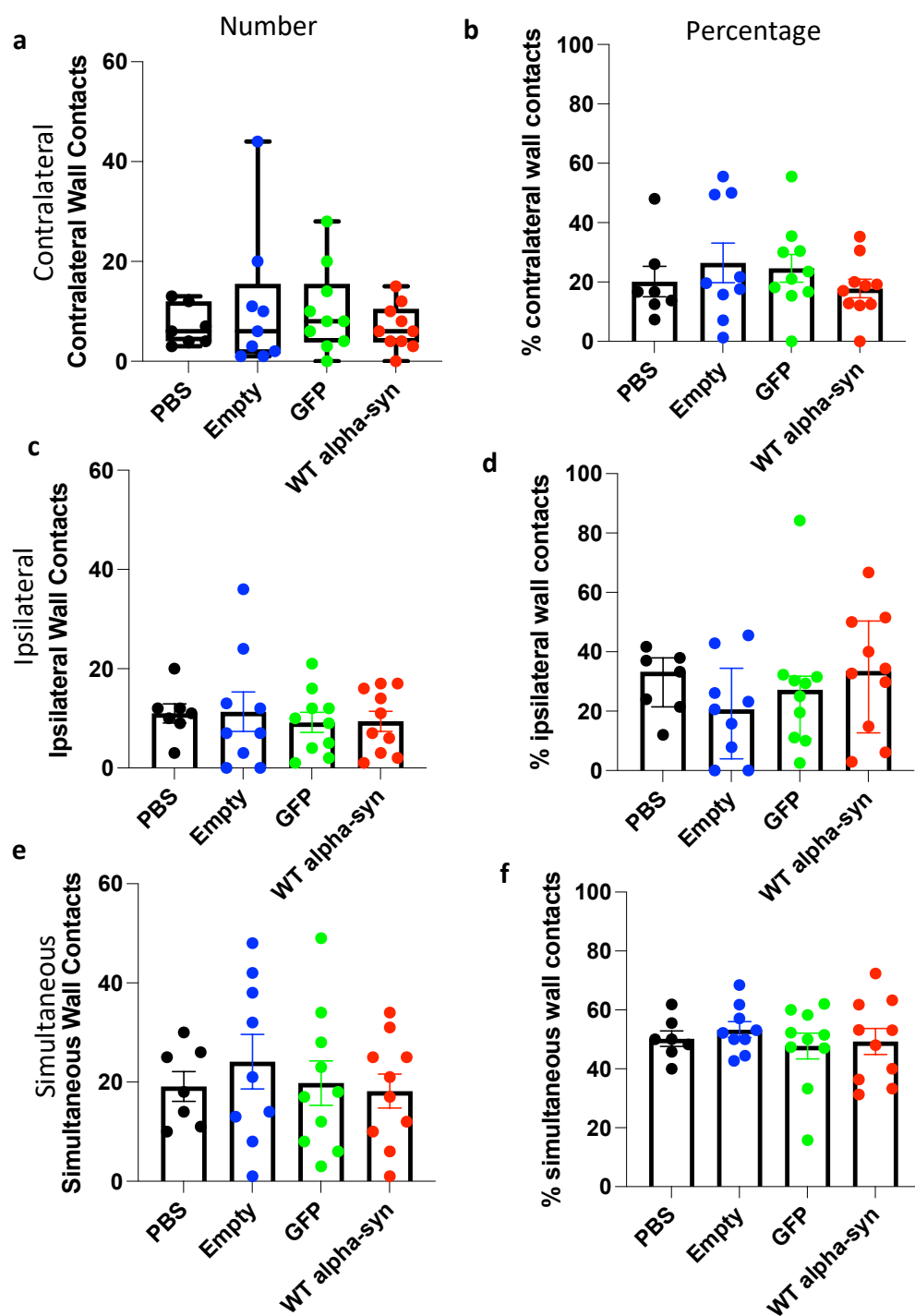


Figure 3.17: Performance in the cylinder test at 14 weeks post-AAV delivery when both sexes were combined. No differences between the treatment groups were identified at 14 weeks post-AAV delivery. Data presented as mean with SEM (b, c, d, e, f) or median with minimum and maximum (a). One-way ANOVA (b, c, d, e, f) or Kruskal-Wallis test (a). n = 7-10.

3.5.3.3 Intranigral injection of AAV5-CBA-alpha-synuclein did not lead to the development of limb use asymmetry at 14 weeks post-AAV delivery when males were analysed alone.

No effects were identified at 14 weeks post-AAV delivery when the males were analysed alone for number of contralateral wall contacts ($H(3) = 1.842, p = 0.6058$), percentage contralateral wall contacts ($H(3) = 0.6650, p = 0.8814$), number of ipsilateral wall contacts ($F(3, 17) = 0.1478, p = 0.9297$), percentage ipsilateral wall contacts ($F(3, 17) = 0.7310, p = 0.5476$), number of simultaneous wall contacts ($F(3, 17) = 0.7133, p = 0.5574$), or percentage simultaneous wall contacts ($H(3) = 2.518, p = 0.4720$) (Figure 3.18).

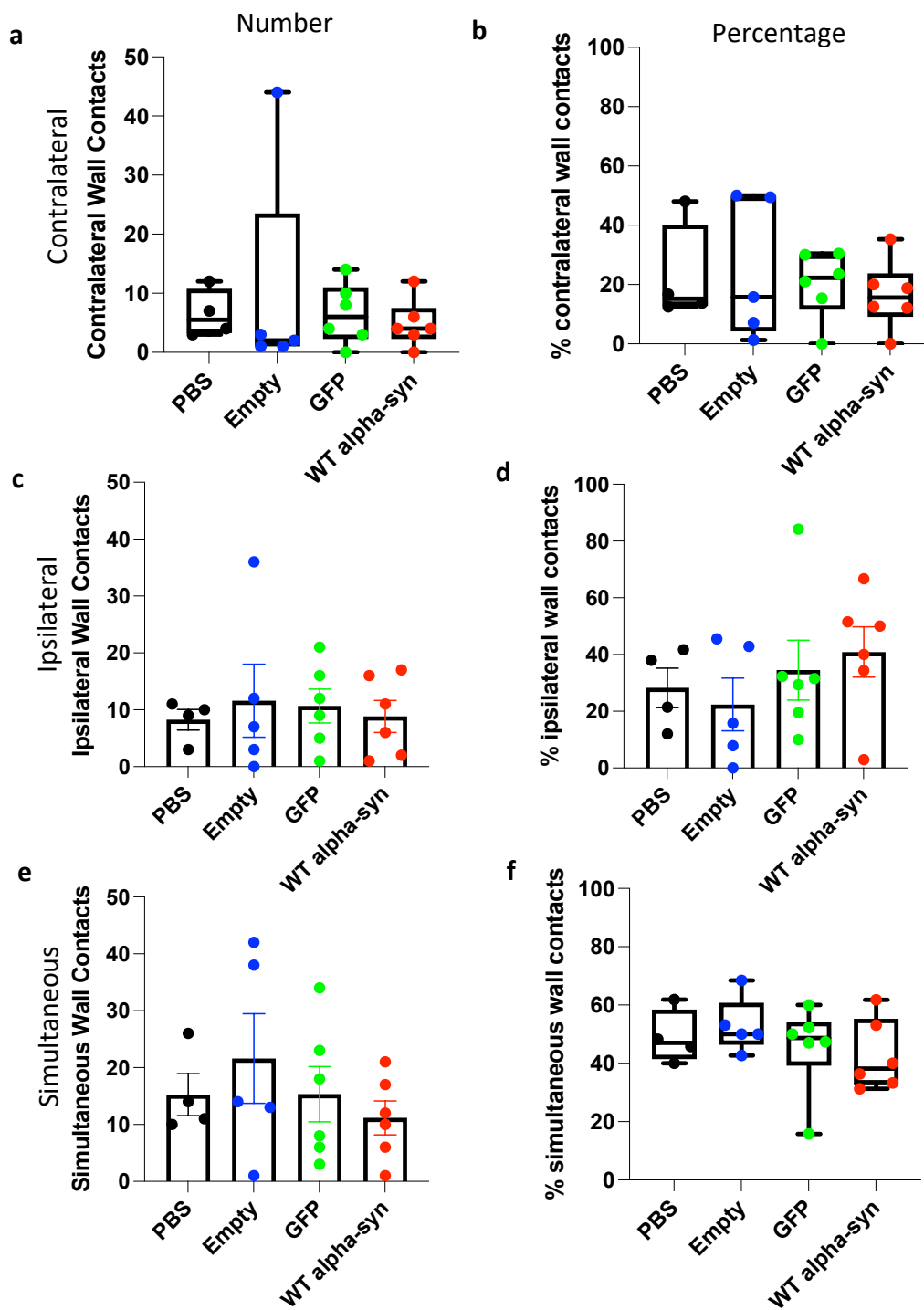


Figure 3.18: Performance in the cylinder test at 14 weeks post-AAV delivery for males alone. No differences between the treatment groups were identified at 14 weeks post-AAV delivery. Data presented as mean with SEM (c, d, e) or median with minimum and maximum (a, b, f). One-way ANOVA (c, d, e) or Kruskal-Wallis test (a, b, f). $n = 4-6$.

3.5.3.4 Intranigral injection of AAV5-CBA-alpha-synuclein did not lead to the development of limb use asymmetry at 14 weeks post-AAV delivery when females were analysed alone.

No effects were identified at 14 weeks post-AAV delivery when the females were analysed alone for number of contralateral wall contacts ($F(3, 11) = 0.8539, p = 0.4933$), percentage contralateral wall contacts ($H(3) = 2.679, p = 0.4762$), number of ipsilateral wall contacts ($F(3, 11) = 0.6904, p = 0.5767$), percentage ipsilateral wall contacts ($F(3, 11) = 1.084, p = 0.3963$), number of simultaneous wall contacts ($F(3, 11) = 0.0702, p = 0.9747$), or percentage simultaneous wall contacts ($F(3, 11) = 0.5429, p = 0.6629$) (Figure 3.19).

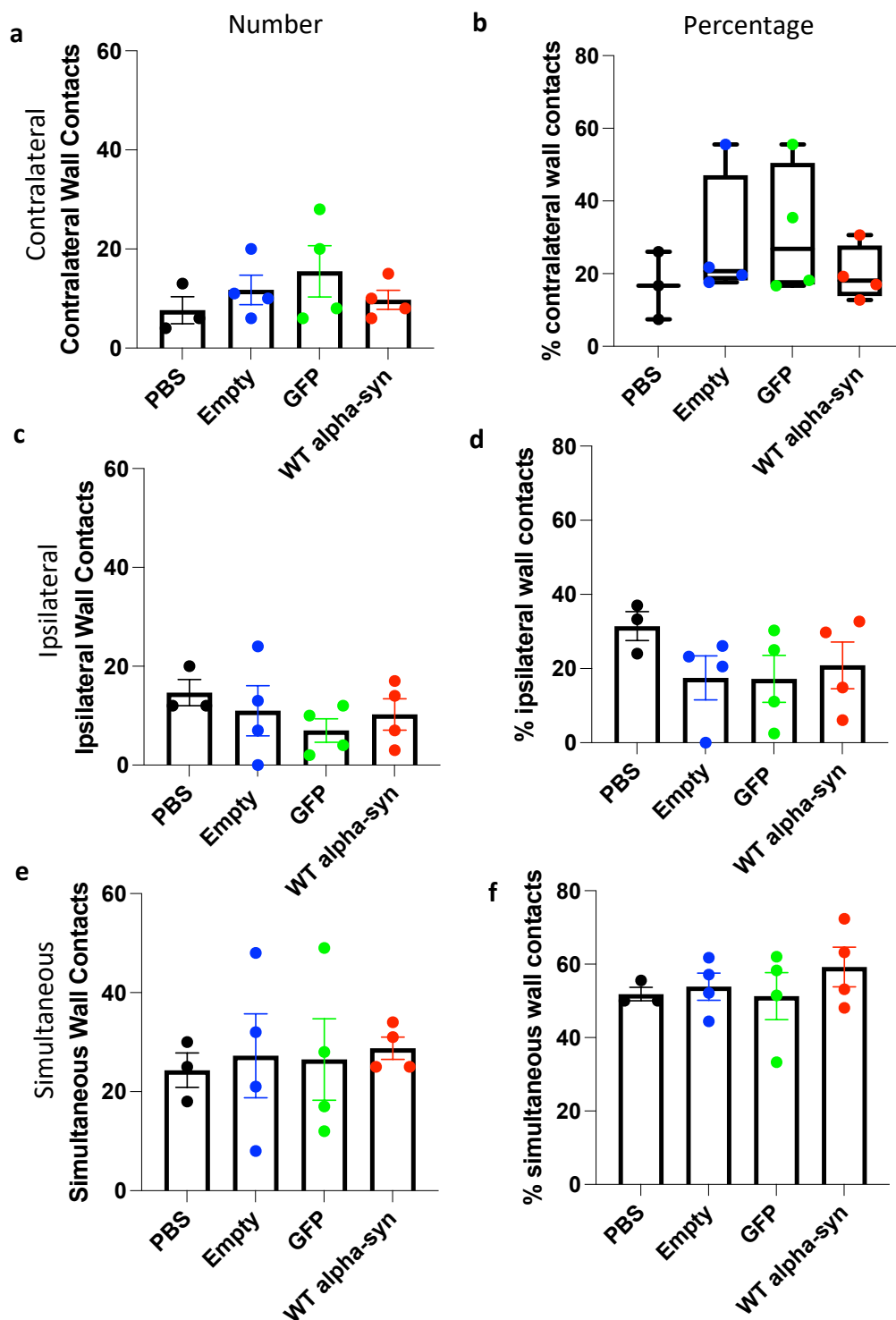


Figure 3.19: Performance in the cylinder test at 14 weeks post-AAV delivery for females alone. No differences between the treatment groups were identified at 14 weeks post-AAV delivery. Data presented as mean with SEM (a, c, d, e, f) or median with minimum and maximum (b). One-way ANOVA (a, c, d, e, f) or Kruskal-Wallis test (b). n = 3-4.

3.5.3.5 Intranigral injection of AAV5-CBA- α -synuclein did not lead to the development of limb use asymmetry as assessed by the limb use asymmetry score.

Limb use asymmetry was assessed pre-AAV delivery and again 14 weeks post-AAV delivery. No effects were identified pre-AAV delivery for both sexes combined ($F(2, 30) = 0.9008, p = 0.4523$), for males alone ($H(3) = 0.2639, p = 0.9667$), or females alone ($F(1, 11) = 1.425, p = 0.2880$). Similarly, at 14 weeks post-AAV delivery no effects were identified for both sexes combined ($F(3, 32) = 0.7693, p = 0.5197$), for males alone ($F(3, 17) = 0.5518, p = 0.6538$), or females alone ($H(3) = 2.842, p = 0.4493$) (Figure 3.20).

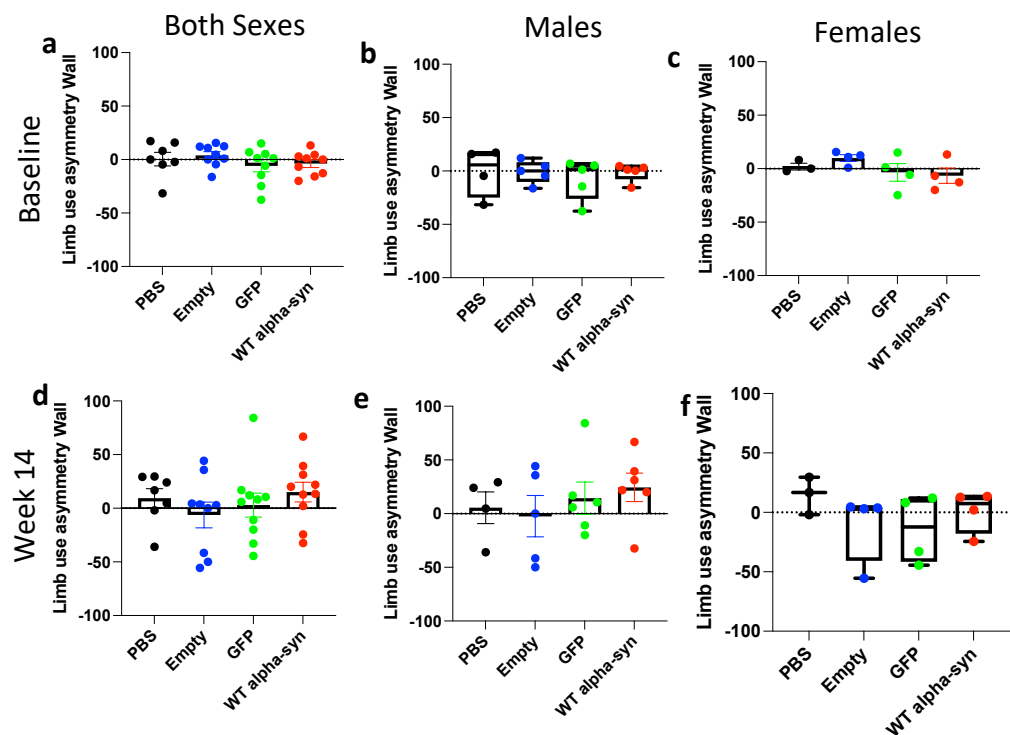


Figure 3.20: Limb use asymmetry in the cylinder test pre-AAV delivery and 14 weeks post-AAV delivery. No differences between the treatment groups were identified at baseline or 14 weeks post-AAV delivery. Data presented as mean with SEM (a, c, d, e) or median with minimum and maximum (b, f). One-way ANOVA (a, c, d, e) or Kruskal-Wallis test (b, f). $n = 7 - 10$ (a, d) both sexes combined, $n = 4 - 6$ (b, e) males, $n = 3 - 4$ (c, f) females.

3.5.4 Amphetamine Rotation Test

3.5.4.1 Intranigral injection of AAV5-CBA-alpha-synuclein did not lead to increases in total rotations at 15 weeks post-AAV delivery over a 60 minute testing period.

Analysis of the total number of rotations over a 60 minute testing period at 15 weeks post-AAV delivery found no effects when both sexes were combined ($H(3) = 5.085$, $p = 0.1657$), for males alone ($H(3) = 4.121$, $p = 0.2487$), or for females alone ($F(3, 11) = 0.6236$, $p = 0.6144$) (Figure 3.21).

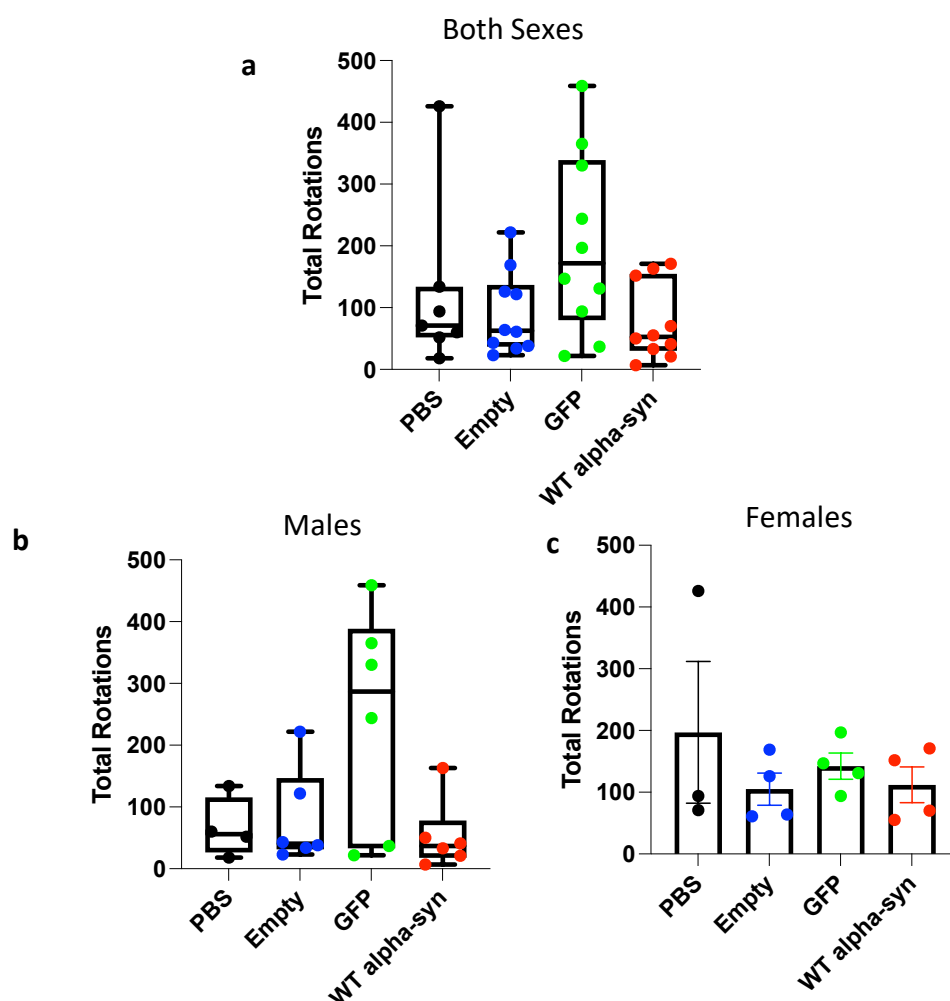


Figure 3.21: Total rotations 15 weeks post-AAV delivery in the amphetamine test over a 60 minute testing period. No differences between the treatment groups were identified at 15 weeks post-AAV delivery. Data presented as median with minimum and maximum (a, b) or mean with SEM (c). One-way ANOVA (c) or Kruskal-Wallis test (a, b). $n = 7 - 10$ (a) both sexes combined, $n = 4 - 6$ (b) males, $n = 3 - 4$ (c) females.

3.5.4.2 Intranigral injection of AAV5-CBA-eGFP but not AAV5-CBA-alpha-synuclein led to increases in ipsilateral rotations at 15 weeks post-AAV delivery over a 60 minute testing period.

Analysis of the total number of ipsilateral rotations at 15 weeks post-AAV delivery over a 60 minute testing period identified an effect for ipsilateral rotations when both sexes were combined ($H(3) = 10.70$, $p = 0.0135$), with post hoc comparisons showing a difference between the AAV-eGFP and AAV-WT alpha-synuclein groups ($p = 0.0091$). No effects were identified when males were analysed alone ($H(3) = 7.394$, $p = 0.0603$), or when females were analysed alone ($H(3) = 3.373$, $p = 0.3621$) (Figure 3.22).

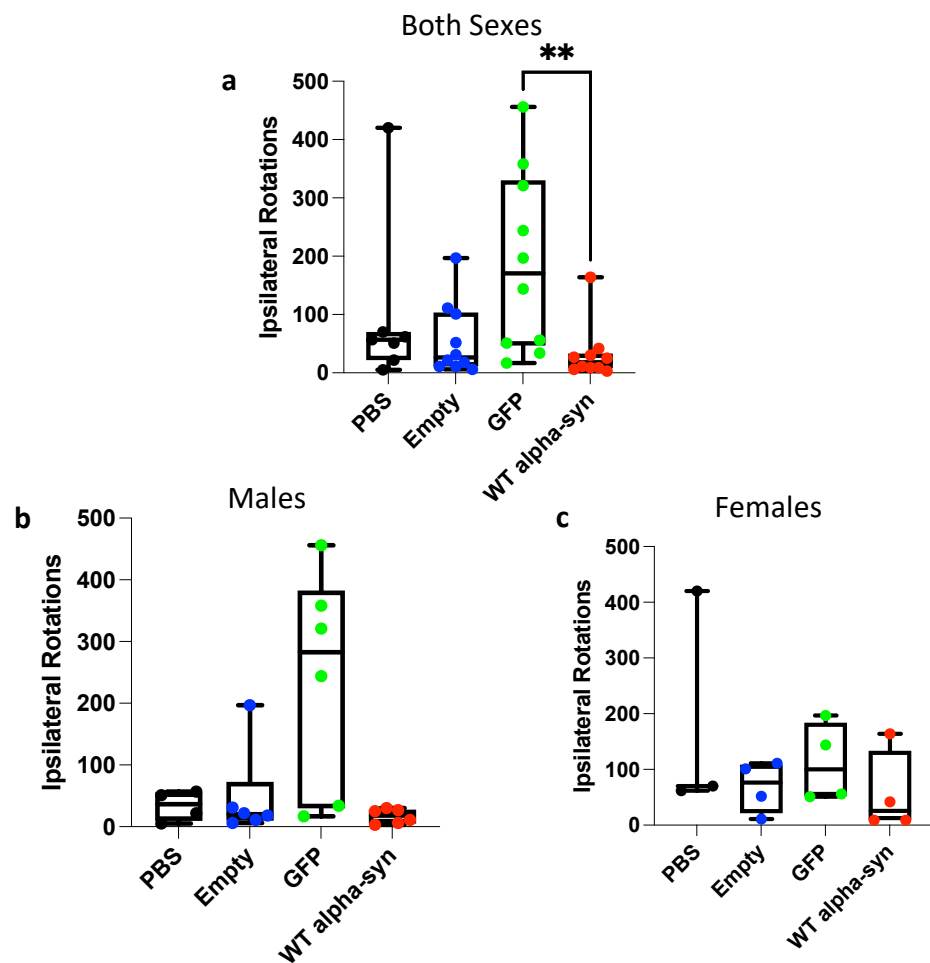


Figure 3.22: Ipsilateral rotations 15 weeks post-AAV delivery in the amphetamine test over 60 minute testing period. Increased ipsilateral rotations were observed in the AAV-eGFP injected animals when both sexes were combined and compared to the AAV-WT alpha-synuclein injected animals. Data presented as median with minimum and maximum. Kruskal-Wallis test followed by Dunn's multiple comparisons test. $n = 7 - 10$ (a) both sexes combined, $n = 4 - 6$ (b) males, $n = 3 - 4$ (c) females. ** $p < 0.01$.

3.5.4.3 Intranigral injection of AAV5-CBA-eGFP but not AAV5-CBA-alpha-synuclein evoked increased ipsilateral rotations in the amphetamine induced rotation test 15 weeks post-AAV delivery from 5 – 30 minutes post amphetamine administration.

The data for ipsilateral rotations was also analysed in two time bins: 5-30 minutes, and 30-60 minutes following amphetamine administration. Analysis of the number of ipsilateral rotations during the 5-30 minute time bin found an effect with both sexes combined ($H(3) = 10.90$, $p = 0.0123$), with post-hoc comparisons identifying a difference between the AAV-eGFP and AAV-WT alpha-synuclein group ($p = 0.0116$). Similarly, an effect was identified when males were analysed alone ($F(3, 18) = 4.334$, $p = 0.0182$), with post-hoc comparisons identifying a difference between the AAV-eGFP and AAV-WT alpha-synuclein group ($p = 0.0235$). No effects were identified when females were analysed alone ($H(3) = 3.933$, $p = 0.2856$) (Figure 3.23).

5 – 30 minutes

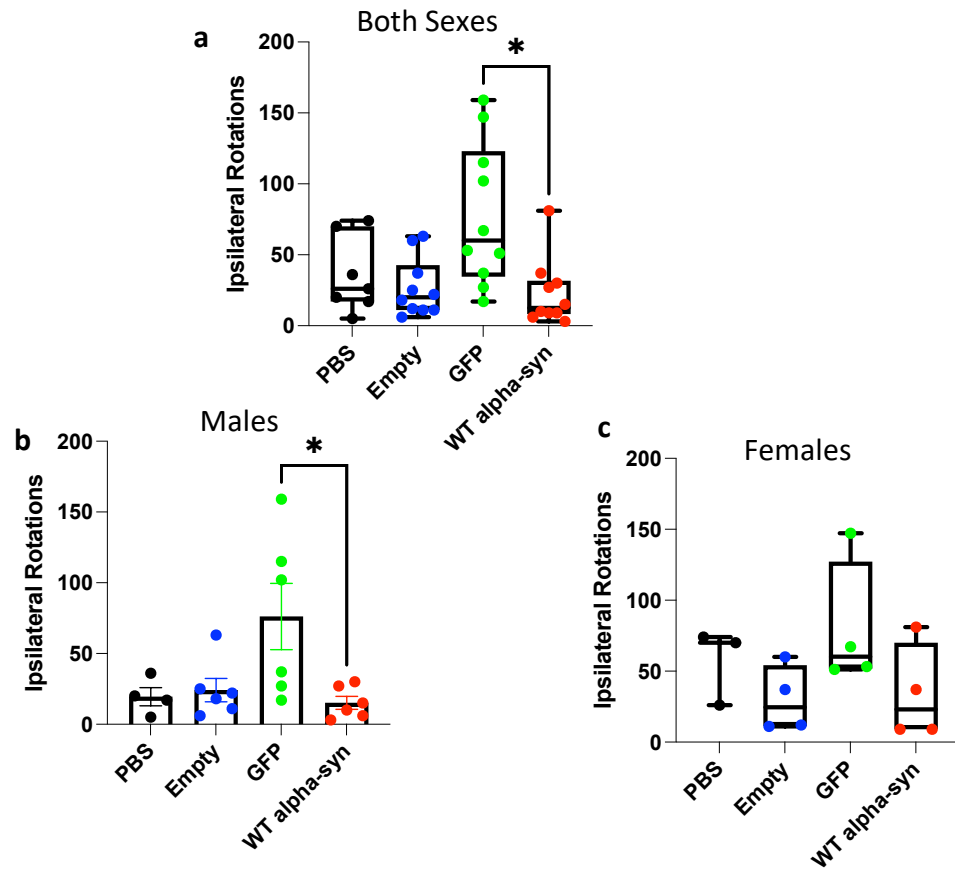


Figure 3.23: Number of ipsilateral rotations at 15 weeks post-AAV delivery 5-30 minutes post-amphetamine administration in the amphetamine induced rotation test. Increased ipsilateral rotations were observed in the AAV-eGFP injected animals when both sexes were combined and when males were analysed alone and compared to the AAV-WT alpha-synuclein injected animals. Data presented as median with minimum and maximum (a, c) or mean with SEM (b). Kruskal-Wallis test followed by Dunn's multiple comparisons (a, c) or one-way ANOVA (b). $n = 7 - 10$ (a) both sexes combined, $n = 4 - 6$ (b) males, $n = 3 - 4$ (c) females. * $p < 0.05$.

3.5.4.4 Intranasal injection AAV5-CBA- α -synuclein did not lead to changes in amphetamine induced ipsilateral rotations at 15 weeks post-AAV delivery from 30 – 60 minutes post amphetamine administration.

Analysis of the number of ipsilateral rotations during 30-60 minutes post amphetamine administration found no effects with both sexes combined ($H(3) = 6.479$, $p = 0.0905$), when males were analysed alone ($H(3) = 7.774$, $p = 0.0509$), or when females were analysed alone ($H(3) = 0.7389$, $p = 0.8833$) (Figure 3.24).

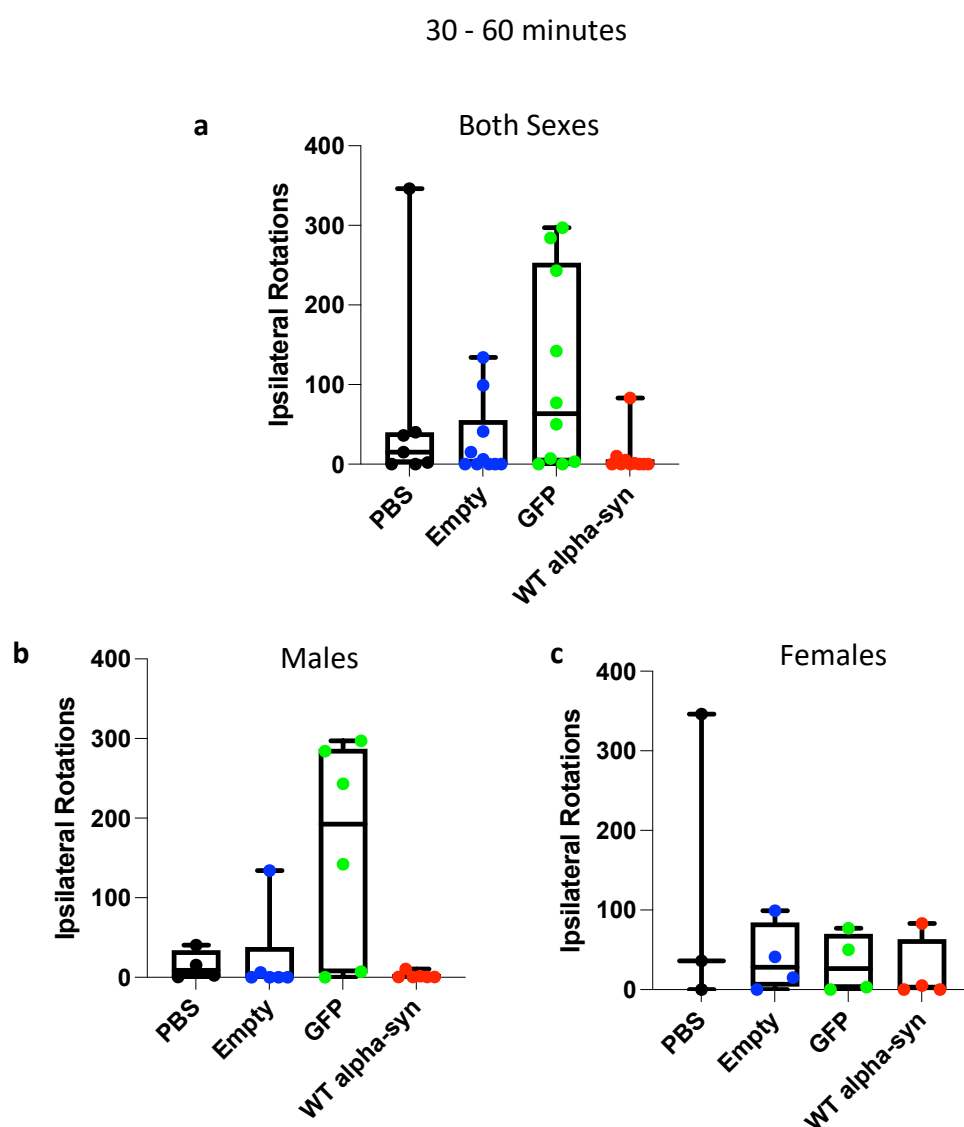


Figure 3.24: Number of ipsilateral rotations at 15 weeks post-AAV delivery in the amphetamine induced rotation test 31-60 minutes post-amphetamine administration. No differences were identified between the treatment groups 31-60 minutes post-amphetamine administration. Data presented as median with minimum and maximum. Kruskal-Wallis test. $n = 7 - 10$ (a) both sexes combined, $n = 4 - 6$ (b) males, $n = 3 - 4$ (c) females.

3.5.5 Open Field Test

3.5.5.1 Intraneural injection AAV5-CBA- α -synuclein did not change distance travelled in metres in the open field test at 13 weeks post-AAV delivery.

The open field test was carried out pre-AAV delivery and again at 13 weeks post-AAV delivery. No effects were identified pre-AAV delivery when both sexes were analysed together ($F(3, 33) = 1.997$, $p = 0.1336$), when males were analysed alone ($F(3, 18) = 2.696$, $p = 0.0767$), or when females were analysed alone ($F(3, 11) = 0.2056$, $p = 0.8904$). Similarly when the data were analysed at 13 weeks post-AAV delivery, no effects were identified when both sexes were analysed together ($F(3, 33) = 0.1835$, $p = 0.9068$), when males were analysed alone ($F(3, 18) = 1.052$, $p = 0.3989$), or when females were analysed alone ($H(3) = 5.763$, $p = 0.1167$) (Figure 3.25).

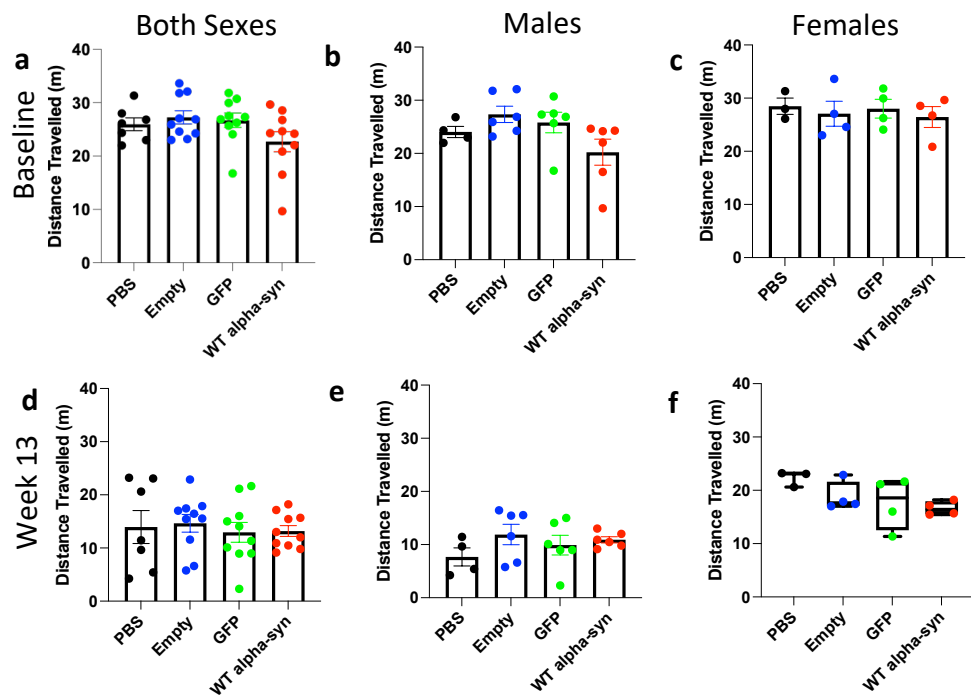


Figure 3.25: Distance travelled in the open field test pre-AAV delivery and 13 weeks post-AAV delivery. No differences were identified between the groups in the open field test at 13 weeks post-AAV delivery. One-way ANOVA (a, b, c, d, e) or Kruskal-Wallis test (f). Data presented as mean with SEM (a, b, c, d, e) or median with minimum and maximum (f). $n = 7 - 10$ (a, b) both sexes combined, $n = 4 - 6$ (c, d) males, $n = 3 - 4$ (e, f) females.

3.5.6 Summary of results – 16 week AAV-WT Study

	Staircase Test (multiple timepoints post-AAV delivery)	Open Field Test 13 weeks post-AAV delivery	Cylinder Test 14 weeks post-AAV delivery	Amphetamine Rotation Test 15 weeks post-AAV delivery	TH immunoreactivity
Both Sexes	No differences	No differences	No differences	Increased ipsilateral rotations in the AAV5-eGFP treatment group when compared with the AAV5-WT-alpha-synuclein treatment group. Evident when full 60 minutes of testing was analysed and when first 30 minutes of testing was analysed.	Not assessed
Males	No differences	No differences	No differences	Increased ipsilateral rotations in the AAV5-eGFP treatment group when compared with the AAV5-WT-alpha-synuclein treatment group. Evident when first 30 minutes of testing was analysed.	
Females	No differences	No differences	No differences	No differences	

Table 3.3: A summary of the results obtained in the 16 week AAV-WT study.

3.6 Discussion

In this study it was shown that the delivery of AAV5-CBA-alpha-synuclein (human WT) to the substantia nigra of rats leads to increased alpha-synuclein immunofluorescence in the injected hemisphere at both 12 and 16 weeks post-AAV delivery. Furthermore, at 16 weeks post AAV-delivery, this increase in immunofluorescence is seen in both male and female rats. Behaviourally, it was found that intranigral injection of AAV5-CBA-alpha-synuclein does not lead to the development of unilateral deficits in either the staircase or cylinder motor behaviour tests at 12 weeks post-AAV delivery, and that increasing this timepoint to 16 weeks post-AAV delivery similarly does not lead to the development of quantifiable motor deficits. Furthermore, through the use of the amphetamine rotation test at 15 weeks post-AAV delivery it was found that the animals injected unilaterally into the substantia nigra with AAV5-CBA-eGFP developed increased ipsilateral rotations, while the AAV5-CBA-alpha-synuclein group did not. Through analysis of the amphetamine test in two time bins, it was found that this effect seemed to be male specific, as in the first 30 minutes of the test the males injected with AAV5-CBA-eGFP demonstrated increased ipsilateral rotations, with no effect observed in the AAV5-CBA-alpha-synuclein group. Separate analysis of females did not demonstrate any increases in amphetamine-induced ipsilateral rotations.

In the 16 week study both male and female rats were included. In humans it is estimated that twice as many males than females will develop PD, suggesting that females are somewhat protected from developing the disease (Gillies et al., 2014, Smith and Dahodwala, 2014). In order to assess if similar protective effects would be identified in rats, a pilot study was conducted with a small group of female rats included. No motor impairments were observed in the AAV5-CBA-alpha-synuclein injected animals, for either males or females. Assessment of males in the AAV5-CBA-eGFP group found increased ipsilateral rotations during the first 30 minutes post-amphetamine injection when compared to the AAV5-CBA-alpha-synuclein injected males. No such motor behavioural differences were identified in the females.

The behavioural findings of the present study suggest that GFP may induce behavioural deficits after prolonged expression. Previous *in vitro* research has found that GFP leads to the induction of apoptosis (Liu et al., 1999, Detrait et al., 2002). Many studies *in vivo* have also investigated the impact of AAV-GFP expression or used it as a control AAV injection. While some research has found little or no cell death or behavioural impairments (Gorbatyuk et al., 2010, Gorbatyuk et al., 2008, Sanchez-Guajardo et al., 2010, Taschenberger et al., 2012, Yamada et al., 2004), there have been other studies which have found that GFP expression can lead to a loss of TH positive immunoreactivity, indicative of dopaminergic neuronal cell death (Klein et al., 2006, Koprach et al., 2010). The titre used in the present study was 9.5×10^{12} vg/ml. In the studies in which TH immunoreactive cell death was observed post GFP injection, the titres used were 5.1×10^{12} gp/ml with 2 μ l injected, and 1×10^{10} vector genomes.

The present study is limited by the lack of quantification of the TH immunoreactive cells in the substantia nigra following AAV delivery. During the processing of the tissue in the 12 week study, the injected hemisphere was not marked on the tissue sections as they were collected. As this study adopted a free-floating immunohistochemistry protocol, it was not possible to determine the injected hemisphere when the sections were being stained for TH quantification with DAB immunohistochemistry. A visual inspection of the tissue sections under the microscope did not indicate any clear differences between the hemispheres for positive TH immunofluorescence in the AAV5-CBA-alpha-synuclein group. While TH positive immunofluorescence could be used to assess TH positive cell counts, this protocol is not currently optimised in the laboratory, and due to the lack of a clear behavioural deficit this approach was not adopted.

Upon review of the behavioural results in the 16 week study, due to the lack of behavioural deficits in the AAV5-CBA-alpha-synuclein group, no further immunohistochemistry or quantification was performed on the substantia nigra. While the present model may be a more long-term model of synucleinopathy, the aim of this study was to develop a model with a more

rapid onset of behavioural deficits. While some studies have found rapid degeneration in models utilising WT human alpha-synuclein delivered through AAV (Kirik et al., 2002, Gorbatyuk et al., 2010, Decressac et al., 2011) this was not the case in the present study. There is considerable variability in the titres used across reported studies necessary to induce degeneration, with some studies utilising higher titres than the present study and others utilising lower titres.

There are many considerations when choosing what type of AAV vector to work with. As well as the promoter and enhancer elements mentioned above, the serotype of the AAV is also important. The present study utilised AAV5, which has been found to transduce neuronal cells in mice and rats (Kirik and Björklund, 2003, Aschauer et al., 2013). Recent studies have utilised hybrid vectors which are composed of the transgene between the inverted terminal repeats (ITRs) of one serotype, for example AAV2, and the capsid genes of a different serotype, for example AAV6, which is provided for *in trans* (Decressac et al., 2011). These hybrid vectors have been found to have enhanced transduction efficiency (McFarland et al., 2009b) and may have been more effective at transducing neuronal cells, leading to a higher expression of alpha-synuclein than that observed in the present study.

To conclude, unilateral injection of AAV5-CBA-alpha-synuclein (2µl; 9.5×10^{12} vg/ml) into the substantia nigra of male or female Wistar rats led to increased alpha-synuclein immunofluorescence in the injected hemisphere when compared to the un-injected hemisphere at both 12 and 16 weeks post-AAV delivery. However this was not sufficient to induce motor behavioural deficits as assessed through the staircase test, cylinder test, or amphetamine rotation test, at 12 or 16 weeks post-AAV delivery. AAV5-CBA-eGFP provoked ipsilateral rotations in male rats at 15 weeks post-AAV delivery following amphetamine administration, with no effects observed in the alpha-synuclein injected animals, or in the females injected with AAV5-CBA-eGFP. While longer term investigation of the behavioural phenotype post-AAV delivery may have

led to the development of behavioural deficits in the longer term, this was beyond the scope of the current project.

4. 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.

4.1 Introduction

Alpha-synuclein, encoded by the synuclein alpha gene *SNCA*, is one of the major components of Lewy bodies, which are a key pathological hallmark of Parkinson's disease (PD) (Nishioka et al., 2020). The variants of *SNCA* can be broadly categorised into two: multiplications, and the missense mutations including A53T, A30P, E46K, and G51D (Deng et al., 2018). The first genetic mutation found to cause a disease phenotype was the A53T mutation (Polymeropoulos et al., 1997). It has been reported that patients with this mutation can have an age of onset of disease between 20 and 85 years, and that these patients present with resting tremor, bradykinesia, postural instability, rigidity, dysarthria, dysphagia, and cognitive impairment. Furthermore, these patients show a good response to levodopa treatment (Xiong et al., 2016).

Many studies have been conducted in order to compare the differences in behavioural phenotype and pathology induced by wildtype (WT) alpha-synuclein AAVs and A53T mutated alpha-synuclein AAVs in preclinical models of disease. Assessment of the alpha-synuclein expression 27 weeks post-intranigral AAV2 delivery of either the WT or the A53T alpha-synuclein found similar pathological changes, including the appearance of alpha-synuclein positive cytoplasmic inclusions and granular deposits, strong alpha-synuclein immunoreactivity in the cytoplasm, swollen alpha-synuclein positive dystrophic and fragmented neurites, and dense, shrunken neuronal perikarya with pyknotic appearance in the substantia nigra (Kirik et al., 2002). While this study

utilised the same serotype (AAV2), the titres were different for the WT and A53T alpha-synuclein, and therefore the results are not directly comparable.

Behavioural comparisons between AAV-WT and AAV-A53T alpha-synuclein have also been made, with behavioural deficits identified in both groups (Kirik et al., 2002) when assessed in the stepping test at eight weeks post-AAV. Furthermore, at four months post-AAV2/6 induced expression of WT or A53T alpha-synuclein behavioural deficits were observed in both alpha-synuclein groups (Gaugler et al., 2012) in the cylinder and amphetamine rotation tests. Analysis of dopamine synthesis, storage and turnover following AAV2-alpha-synuclein WT or AAV2-A53T-alpha-synuclein identified decreased striatal dopamine concentrations as well as reduced striatal TH enzyme activity at eight weeks post-AAV delivery which remained unchanged at 27 weeks post-AAV delivery (Kirik et al., 2002).

While the studies described above find no differences between WT and A53T alpha-synuclein expression mediated through AAV and associated behavioural deficits, the findings of Chapter 3 indicate that perhaps the pathology and motor behavioural deficits induced by AAV-WT-alpha-synuclein can take quite some time to develop. Research utilising AAV1/2 mediated expression of A53T alpha-synuclein has shown the development of PD-like pathology and motor behavioural deficits as early as 3 weeks post-AAV, as well as alpha-synuclein expression (Koprich et al., 2010). This expression is accompanied by reduced TH positive immunoreactivity indicative of dopaminergic neuronal cell loss, however this was also observed in the AAV1/2-GFP injected substantia nigra.

In the study described above, Female Sprague Dawley rats received a single unilateral intranigral injection of AAV1/2 empty vector, AAV1/2 GFP, or AAV1/2 human A53T alpha-synuclein (5.1×10^{12} viral genomes/ml) (Koprich et al., 2010). Through a proteinase K digestion assay it was found that there was a large expression of alpha-synuclein aggregates in the A53T group, which were resistant to digestion, and therefore could be viewed as insoluble aggregates, which are a key pathological hallmark of PD. A further study was conducted in

order to assess the impact of different titres of AAV1/2-A53T human alpha-synuclein on motor behaviour and dopaminergic neuronal integrity at both three and six weeks post-AAV delivery (Koprach et al., 2011). The titres used in this study were 1:3 and 1:10 of the titre used in the previous experiment discussed above (Koprach et al., 2010). Both titres of AAV1/2-A53T alpha synuclein were found to lead to forelimb use asymmetry in the cylinder test at three weeks post-AAV delivery. Interestingly, at six weeks post-AAV delivery, the motor behavioural deficit persisted only in the 1:3 dilution group, and was no longer present in the 1:10 dilution group. Reduced TH immunoreactivity was also evident in both titre groups for the AAV1/2-A53T alpha synuclein treated animals at three weeks post-AAV, while at six weeks post-AAV delivery this was only observed in the AAV1/2-A53T alpha-synuclein 1:3 dilution group (Koprach et al., 2011).

Reduced TH positive immunoreactivity in the substantia nigra and motor behavioural deficits have also been reported at four weeks post-AAV2/9 A53T delivery (Bourdenx et al., 2015). Assessment of TH and dopamine transporter (DAT) immunoreactivity in the substantia nigra found that from one week post-delivery of AAV2/9-A53T-alpha-synuclein the immunoreactivity of these markers was decreased, with a plateau of reduction reached at four weeks post-AAV delivery. This decrease was associated with progressive dopaminergic neuronal cell death, which ranged from approximately 50% at one week post-AAV delivery to approximately 80% at 16 weeks post-AAV delivery. Human alpha-synuclein was first observed in the substantia nigra at 72 hours post-AAV delivery, with this expression level increasing up to eight weeks post-AAV delivery, where it peaked. Behavioural analysis found that a decrease in the number of adjusting steps in the stepping test evident at four weeks post-AAV2/9-A53T-alpha-synuclein delivery when compared with 'sham' injected animals, and this continued to the end of the experiment at 16 weeks post-AAV delivery. Furthermore, decreased spontaneous activity was observed in the AAV2/9-A53T-alpha-synuclein injected animals from eight weeks post-AAV delivery to the end of the experiment at 16 weeks post-AAV delivery.

The impact of two injections of AAV2-A53T-alpha-synuclein into the substantia nigra was assessed in female Sprague Dawley rats (Chung et al., 2009). Alpha-synuclein expression was observed in the substantia nigra and the striatum as early as four weeks post-AAV2-A53T-alpha-synuclein delivery, which persisted to the final endpoint measured at 24 weeks-post-AAV delivery. In contrast to Koprach and colleagues (2011) study, reduced TH positive immunoreactivity was not observed until 17 weeks post-AAV delivery in the AAV2-A53T-alpha-synuclein group, at which time an approximately 32% dopamine neuronal cell loss was observed, which increased to approximately 53% at 24 weeks post-AAV delivery. Similarly, other studies have not observed reduced TH immunoreactivity until 24 weeks post-AAV delivery (Rocha et al., 2015).

Taken together, the studies mentioned above suggest that the expression of A53T human alpha-synuclein can be induced through the injection of AAV vectors in rats. While the data for reduced TH immunoreactivity and associated dopaminergic neuronal cell loss is variable, reduced TH immunoreactivity was observed at multiple time points post-AAV delivery, including as early as three weeks post-AAV delivery. Many studies also report behavioural deficits, also recorded as early as three weeks post-AAV delivery. The present study aimed to assess the impact of delivery of AAV1/2-CMV-A53T alpha-synuclein (human) to the substantia nigra of rats and compare this to the impact of delivery of sterile PBS and AAV1/2-CMV-Null/Empty, or the impact of the delivery of AAV1/2-CMV-Null/Empty alone at multiple time points post-AAV delivery, on behavioural motor performance and TH positive immunoreactivity.

Motor behaviour is assessed by performance in the cylinder test, stepping test, amphetamine test and staircase test. The AAV1/2 vector was chosen in this study as it has been found to induce expression of alpha-synuclein and provoke a loss in TH positive immunoreactivity as early as three weeks post-AAV delivery (Koprach et al., 2010). While AAV5 was shown to lead to alpha-synuclein immunofluorescence at 12 and 16 weeks post-delivery, this immunofluorescence was not as strong as had been anticipated, even though AAV5 has a strong tropism for neurons. As AAV5 also shows a tropism for

astrocytes it is possible that this diluted its impact on neurons. Furthermore, through the chimeric approach, the benefits of both AAV1 (strong ability to penetrate brain tissue) and AAV2 (neuronal tropism and the ability to produce high titres) can be exploited. AAV1 has been shown to have tropism for neurons, oligodendrocytes and astrocytes, while AAV2 shows tropism only for neurons.

4.2 Study Aims and Objectives

The aim of this study was to confirm expression of alpha-synuclein in the rat substantia nigra and characterise resultant motor deficits and changes in TH immunoreactivity following AAV1/2 mediated delivery of A53T human alpha-synuclein. To this end, rats received a single unilateral injection of AAV1/2-CMV-A53T alpha-synuclein (human) (2µl; 5.1×10^{12} viral genomes/ml) to the substantia nigra, and contralateral motor deficits, intra-nigral alpha-synuclein immunofluorescence, and TH positive immunoreactivity were assessed at 3.5, 6.5 and 13 weeks post-AAV delivery.

Specifically, we anticipated that a single unilateral injection of AAV1/2-CMV-A53T alpha-synuclein (human) would lead to clear overexpression of alpha-synuclein immunofluorescence at 3.5, 6.5 and 13 weeks post-AAV delivery, and this would be coupled with the emergence of a time dependent unilateral motor dysfunction associated with reduced TH immunoreactivity and dopamine neuronal loss in the substantia nigra.

4.3 Methods

4.3.1 Justification of Experimental Design

AAV1/2 is chosen as serotype as previous research has found it to be efficient in transducing cells in the substantia nigra (Koprach et al., 2011), as well as their availability from Vigene Biosciences. A53T alpha-synuclein is investigated as this mutation is associated with the development of PD in humans and it can be delivered to the rodent brain through the use of AAV vectors. The titres utilised have been validated previous researchers (Koprach et al., 2011) and are therefore adopted in the present study. Empty vector is chosen as control in order to control for the impact of expression of an AAV vector. GFP is included in the 3.5 week study in order to explore the impact of the expression of a foreign protein through AAV vectors. 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. PBS was included as a control for the impact of injection. 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 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 initial 3.5 week timepoint is chosen based on the literature reviewed above, with the findings of the 3.5 week study informing the decision to extend the following two studies to a 6.5 and 13 week timepoint.

4.3.2 Experimental Timeline

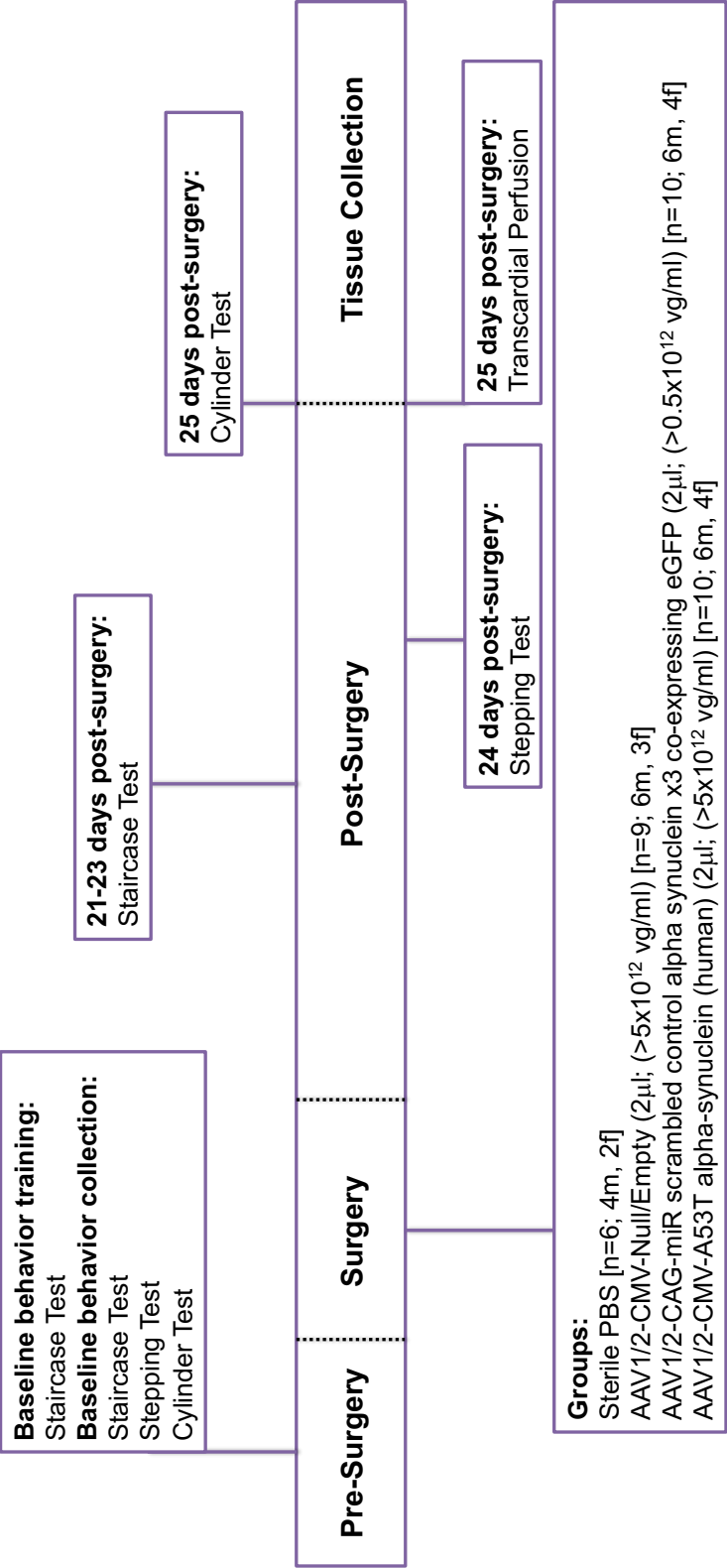


Figure 4.1: Experimental timeline for the *in vivo* phase of the 3.5 week AAV-A53T alpha-synuclein study including behavioural testing timepoints, treatment groups, and tissue collection timepoint.

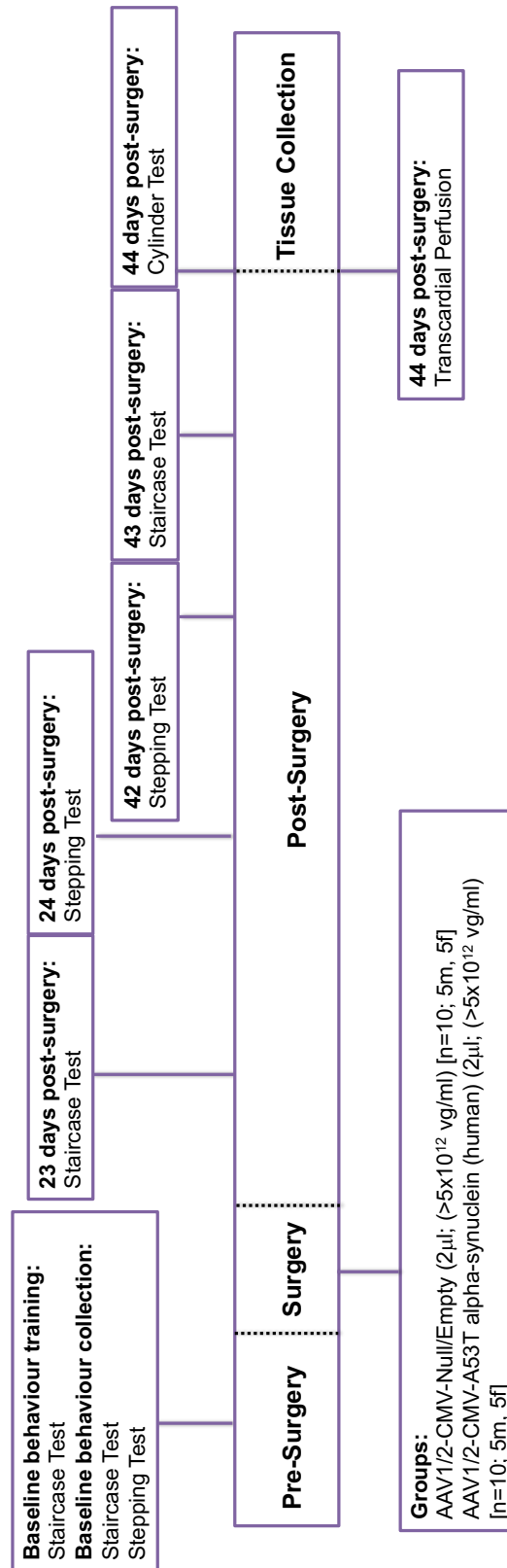


Figure 4.2: Experimental timeline for the *in vivo* phase of the 6.5 week AAV-A53T alpha-synuclein study including behavioural testing timepoints, treatment groups, and tissue collection timepoint.

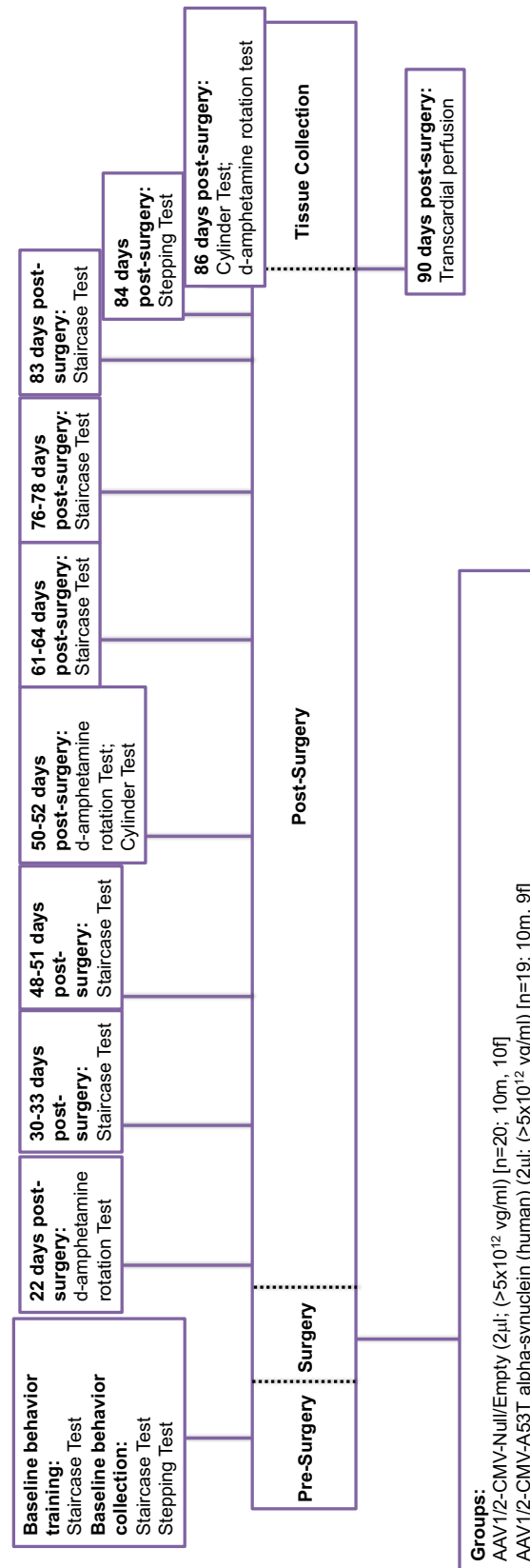


Figure 4.3: Experimental timeline for the *in vivo* phase of the 13 week AAV-A53T alpha-synuclein study including behavioural testing timepoints, treatment groups, and tissue collection timepoint.

4.3.3 Experimental Design

All rats were habituated to the housing unit for at least 2 weeks before any experimental procedures commenced. All rats received 3 x 10 minute training sessions in the staircase test in order to establish proficiency in the test. Rats received a single unilateral injection into the substantia nigra as outlined in Table 4.1 below.

Sex	Virus titre and injection volume	Coordinates
Male	- Sterile PBS [2µl] - AAV1/2-CMV-A53T alpha-synuclein (human) [2µl; (5.1x10¹² vg/ml)] GD1001-RV - AAV1/2-CMV-Null/Empty [2µl; (5.1x10¹² vg/ml)] GD1004-RV - AAV1/2-CAG-miR scrambled control-alpha synuclein co-expressing eGFP [2µl; (0.5x10¹² vg/ml)] GD1009-RV-C	AP -5.3mm from bregma ML +2.0mm from bregma DV -8.5mm from skull
Female	- Sterile PBS [2µl] - AAV1/2-CMV-A53T alpha-synuclein (human) [2µl; (5.1x10¹² vg/ml)] GD1001-RV - AAV1/2-CMV-Null/Empty [2µl; (5.1x10¹² vg/ml)] GD1004-RV - AAV1/2-CAG-miR scrambled control alpha synuclein co-expressing eGFP [2µl; (0.5x10¹² vg/ml)] GD1009-RV-C	AP -5.3mm from bregma ML +2.0mm from bregma DV -7.5mm from skull

Table 4.1: Virus Titre, Injection Volumes and Coordinates for the A53T studies
 For the 3.5 week study all 4 treatment groups were included. In the 6.5 and 13 week studies only the AAV1/2-CMV-Null/Empty and AAV1/2-CMV-A53T alpha-synuclein (human) groups were included.

Behavioural testing in the staircase test and stepping test was carried out prior to surgery and AAV-delivery as a baseline measure of performance. Baseline performance in the cylinder test was assessed in the 3.5 and 13 week study. Post-AAV delivery behavioural testing was carried out as outlined in the experimental timelines presented in Figures 4.1, 4.2 and 4.3.

4.3.4 Adaptations to protocols

All behavioural testing, immunohistochemistry and immunofluorescence was carried out as described in Chapter 2, with the following exceptions.

4.3.4.1 Staircase Test

The testing protocol for the AAV-A53T 13 week study is modified in comparison to the other studies. At nine weeks post-AAV delivery the staircase test is performed over two consecutive days. On the first day, only the side of the staircase ipsilateral to the injection site is baited. On the second day of testing, only the side of the staircase contralateral to the injection site is baited. This is a modified version of the staircase test known as the side test (Montoya et al., 1991). This version of the test is employed in order to evaluate if any side differences observed are due to the animal directing its attention or effort primarily to one side as opposed to greater motor skill on one side over the other.

At 12 weeks post-AAV delivery, when the rats were exposed to the staircase test for the final time, only the side of the staircase contralateral to the injection site is baited in order to force the limb contralateral to the injection to perform.

The staircase test is video recorded at baseline, 76-78 days post-AAV delivery and 83 days post-AAV delivery. For all other time points assessed the trial is not video recorded and data are presented as number of pellets retrieved alone. Percentage success is only presented where the trials were video recorded.

4.3.4.2 Stepping Test

For baseline collection in the 3.5 week and 6.5 week AAV-A53T studies each animal is exposed to only one trial per limb in each direction. For subsequent post-surgical testing, and for baseline data collection in the 13 week AAV-A53T study the animals are exposed to 3 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 3 times for each limb in each direction it allows the animal to become more comfortable and less rigid in the restraint.

4.3.4.3 Cylinder Test

For the 3.5 week AAV-A53T study the animals are exposed to the apparatus twice, once to record baseline behaviour and then at 25 days post-AAV delivery. In the 6.5 week AAV-A53T study, the animals are only exposed to the apparatus once, at 44 days post-AAV delivery in order to mitigate the decrease in rearing behaviour which is observed in previous experiments. For both of these studies the behaviour of the animals is recorded from above, which allows for scoring of wall contacts but does not allow for scoring of floor landings.

In the 13 week AAV-A53T study the animals are exposed to the apparatus twice, at 50-52 days post-AAV delivery and 86 days post-AAV delivery. A number of modifications are made to the way the test was performed for this study. Firstly, 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 to allow them more space to move freely. The females are tested in a cylinder of dimensions (19cm diameter x 50cm height).

The second modification made to the test is the way in which it was recorded. Previously, all recordings were made from above which allowed for scoring of the wall placements but not of the floor landings. In order to allow scoring of both, which would give a clearer dataset, the recordings are now made from the side. This involves setting up two cameras on either side of the cylinder in order to record all the wall placements and floor landings of the animal.

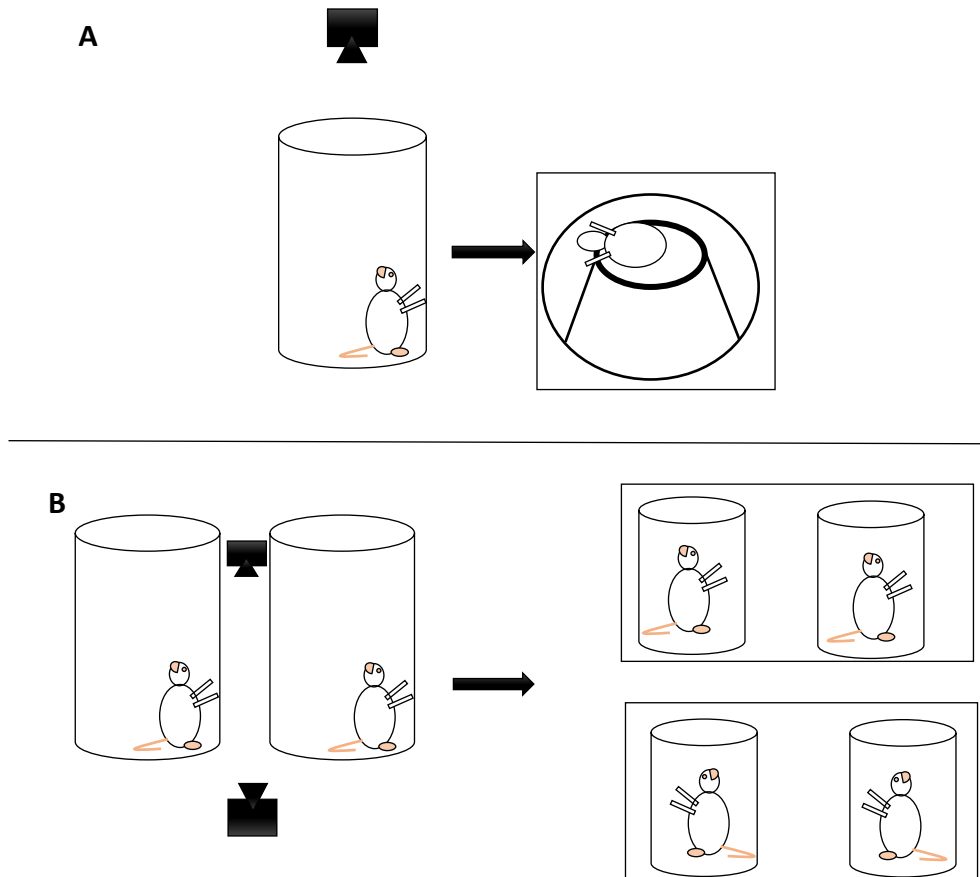


Figure 4.4: Illustration of the modifications made to the cylinder test between the 6.5 and 13 week AAV-A53T studies. A) The set-up of the cylinder test up to and including the 6.5 week AAV-A53T study. The placement of the camera on top of the cylinder allows for a narrow field of view for the observer to assess behaviour during testing with very poor visibility for any floor contacts. B) The set-up of the cylinder test from the 13 week AAV-A53T study onwards. Recording from both sides of the cylinder allows for a clearer view of all wall contacts made by the animal during testing, as well as the floor placements made.

4.3.4.4 Amphetamine rotation test

The duration of the test, as well as the apparatus within which the test were conducted is adapted throughout the 13 week AAV-A53T study. During the first exposure to the amphetamine rotation test the animals are exposed to the same set up as outlined in Chapter 2, and tested for one hour post-amphetamine injection. The duration of the rotation test is then altered during testing at 50-52 days post-AAV delivery, as it was observed that the amphetamine induced effects persisted longer than the testing duration. At the 50-52 days post-AAV delivery the duration of the test is either 60 minutes, 75 minutes or 90 minutes, due to the persistent effects being observed after 60 minutes. The data from this timepoint is expressed as ipsilateral and contralateral rotations per minute only, as it is not possible to make a direct comparison between the animals with different test durations by presenting total ipsilateral and total contralateral rotations. The behaviour of the animals is recorded for 120 minutes for the AAV-A53T 13 week study 86 days timepoint.

As opposed to using open top cages to perform the test as at the 22 days post-AAV delivery timepoint, the cylinders used in the cylinder test are employed for the second and third exposures to the amphetamine rotation test. This alteration is made as it was observed that in the open top cages the animals have a tendency to hold onto the bars of the cage and this prevents the rotational behaviour. Many previous studies using the cylinder test perform the test in a circular apparatus as opposed to a rectangular cage (Robinson et al., 1994, Ungerstedt and Arbuthnott, 1970) and therefore we are making this adaptation. The behaviour of the animals is recorded with a camcorder and scored manually at the 22 days post-AAV delivery timepoint. For the 50-52 days post-AAV delivery and 86 days post-AAV delivery timepoints the behaviour is recorded, tracked and scored using ANYmaze automated software.

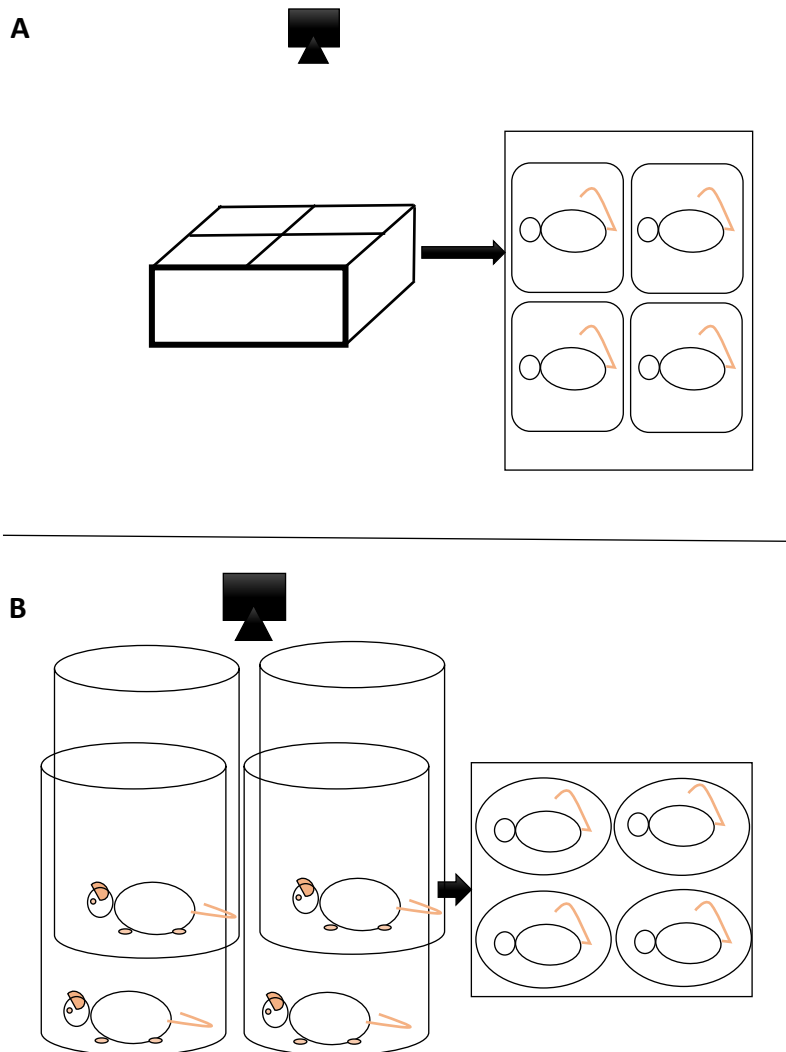


Figure 4.5: Illustration of the modifications made to the amphetamine rotation test between the 22 days post-AAV delivery timepoint and the subsequent testing in the amphetamine rotation test. A) The set-up of the amphetamine rotation test at 22 days post-AAV delivery in the 13 week AAV-A53T study. The use of rectangular open top cages allowed for the animal to hold onto the bars of the cage, interfering with rotational behaviour. B) The set-up of the amphetamine rotation test for the 50-52 days post-AAV delivery and 86 days post-AAV delivery timepoints. The use of cylinders prevents the animal from holding onto any surface in order to decrease the rotations.

4.3.4.5 Immunohistochemistry - Tissue Processing

All brain slices are collected in 96 well plates for the 6.5 and 13 week studies.

4.3.4.6 Immunohistochemistry - Image Analysis

For the 3.5 week study, one 40X image per hemisphere from the substantia nigra is acquired at the same location in each hemisphere. The number of TH immunoreactive cells are manually counted using the cell counter tool in ImageJ.

For the 6.5 and 13 week A53T study two 40X images of the substantia nigra per hemisphere are acquired in the same locations in each hemisphere and the number of TH immunoreactive cells per image 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.

The analysis performed does not adopt an unbiased stereology approach, and the cell count numbers are therefore not absolute. The number of cells illustrated throughout this chapter are based on counting the cells present in a two-dimensional image of the injected hemisphere, and expressing this over the number of cells counted in a one-dimensional image of the un-injected hemisphere.

4.4 Results – 3.5 week AAV-A53T Study

4.4.1 Alpha-synuclein immunofluorescence

4.4.1.1 Increased alpha-synuclein immunofluorescence was observed in the injected hemisphere when compared to the un-injected hemisphere of the substantia nigra for both sexes combined and when females were analysed alone.

Comparison of the injected and un-injected substantia nigra of the animals in the AAV-A53T groups identified a difference between the sides with both sexes combined ($t(9) = 4.353$, $p = 0.0024$), and for females alone ($t(3) = 4.237$, $p = 0.0241$), with no difference identified for males alone ($t(4) = 2.367$, $p = 0.0770$) (Figure 4.6).

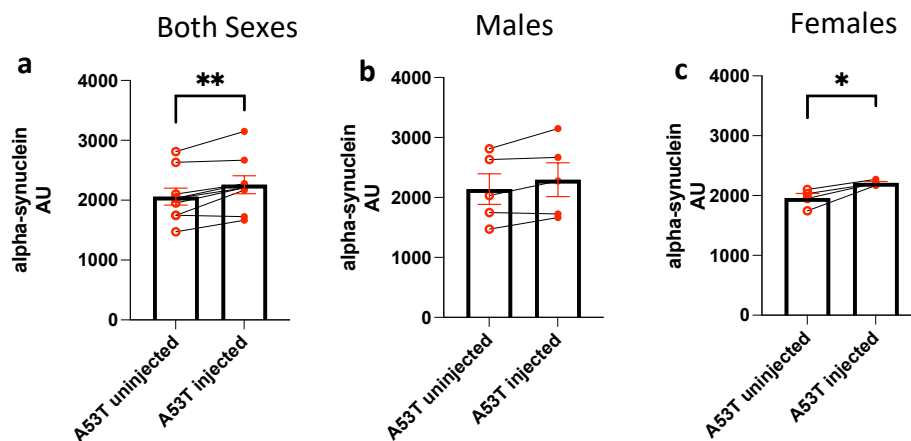


Figure 4.6: Comparison of the injected and un-injected hemispheres of the substantia nigra of the animals in the AAV-A53T group. Increased alpha-synuclein immunofluorescence was identified in the injected hemisphere when both sexes were combined (a) and when females were analysed alone (c), with no differences identified for males alone (b). Data presented as mean with SEM. Paired t-test. AU = arbitrary units. $n = 10$ (a), $n = 6$ (b), $n = 4$ (c). * $p < 0.05$, ** $p < 0.01$.

4.4.1.2 Review of the AAV-alpha-synuclein injected animals found varying alpha-synuclein immunofluorescence across the substantia nigra and between animals at 3.5 weeks post-AAV delivery.

Alpha-synuclein immunofluorescence was assessed in the brains of AAV-alpha-synuclein treated animals in both the injected and un-injected hemispheres. A number of sections, each 175µm apart, were analysed for alpha-synuclein immunofluorescence intensity. Data were assessed as the alpha-synuclein immunofluorescence in the un-injected side divided by the alpha-synuclein immunofluorescence in the injected side. Due to the serial nature of the tissue sectioning and immunofluorescence staining on a series of sections, it is possible the exact location where the AAV was delivered was not assessed in every animal. Animal 27 was found to express alpha-synuclein immunofluorescence under 100% of the un-injected hemisphere, when the injected hemisphere was expressed as a percentage of the un-injected hemisphere, and this animal was removed from all behavioural analysis.

Animal	Sex	Expression over 100% (number of sections)
3	Male	5/9
13	Female	7/8
21	Male	5/6
27	Male	2/9
31	Female	8/10
4	Male	6/8
14	Female	10/10
22	Male	4/9
28	Male	8/10
35	Female	6/8

Table 4.2: Analysis of alpha-synuclein immunofluorescence in the substantia nigra in the injected and un-injected hemisphere of animals in the AAV-A53T group. Expression over 100% represents alpha-synuclein immunofluorescence in the injected hemisphere expressed as percentage of the un-injected side, that is greater than 100%. The data represented in this table are taken from a number of sections from the substantia nigra.

Representative images of alpha-synuclein and TH positive immunofluorescence in the substantia nigra of male and female AAV1/2-CMV-A53T alpha-synuclein treated animals (Figure 4.7).

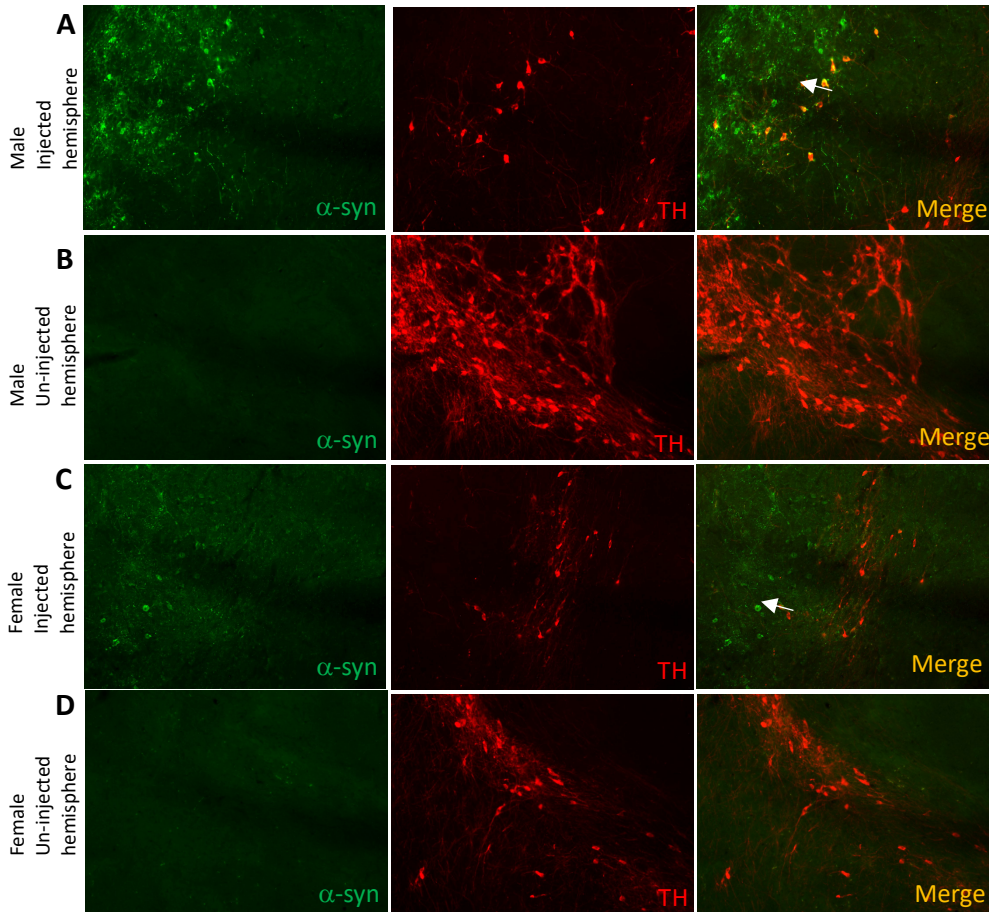


Figure 4.7: Representative images of alpha-synuclein and TH immunofluorescence in the substantia nigra 3.5 weeks post-delivery. (A) AAV-A53T alpha-synuclein injected male injected hemisphere, (B) AAV-A53T alpha-synuclein injected male un-injected hemisphere, (C) AAV-A53T alpha-synuclein injected female injected hemisphere, (D) AAV-A53T alpha-synuclein injected female un-injected hemisphere. First column represents alpha-synuclein immunofluorescence, second column represents TH immunofluorescence, third column represents merged images of the first 2 columns. Images acquired at 20X. Arrow indicating co-expression of alpha-synuclein and TH.

4.4.3 Cylinder Test

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

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 ($F(3, 30) = 0.1183, p = 0.9496$). Similarly, no differences were identified when males and females were analysed separately. Data presented in Appendix II.

4.4.3.2 Intranigral injection of AAV1/2-CAG-miR scrambled control alpha-synuclein co-expressing eGFP but not AAV1/2-CMV-A53T alpha-synuclein led to limb use asymmetry at 25 days post-AAV delivery.

Analysis of performance in the cylinder test at 25 days post-AAV delivery identified an effect of treatment on the number of contralateral wall contacts ($H(3) = 8.763, p = 0.0326$). Post-hoc comparisons found that the AAV-GFP group had less contralateral wall contacts than the AAV-empty group ($p = 0.0421$). No effects of treatment were found for percentage contralateral wall contacts ($F(3, 30) = 2.652, p = 0.0667$), number of ipsilateral wall contacts ($F(3, 30) = 1.748, p = 0.1784$), percentage ipsilateral wall contacts ($F(3, 30) = 2.912, p = 0.0506$), number of simultaneous wall contacts ($H(3) = 2.305, p = 0.5116$), or percentage simultaneous wall contacts ($F(3, 30) = 0.5394, p = 0.6590$) (Figure 4.8).

Analysis of performance in the cylinder test for males alone found no differences between the treatment groups (data not presented). Due to the low sample size per groups no statistical analysis were performed for females alone.

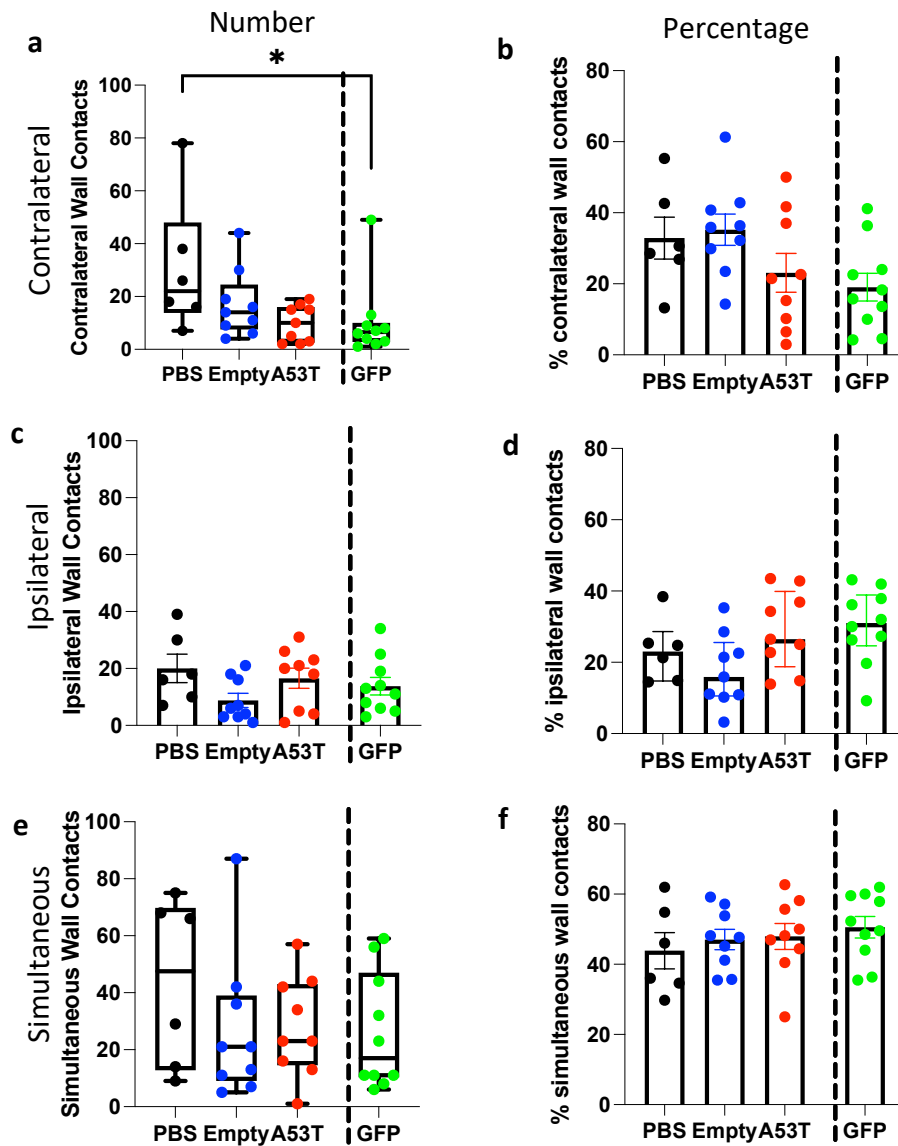


Figure 4.8: Performance in the cylinder test 25 days post-AAV delivery for both sexes combined. Analysis of performance in the cylinder test 25 days post-AAV delivery found that the AAV-GFP group had lower contralateral wall contacts than the PBS group. Data presented as mean with SEM (b, c, d, f) or median with minimum and maximum (a, e). One-way ANOVA (b, c, d, f) or Kruskal-Wallis test (a, e). $n = 6-10$. * $p < 0.05$.

4.4.3.3 Limb use asymmetry was not identified at baseline or at 25 days post-AAV delivery as assessed by the limb use asymmetry score.

Limb use asymmetry was assessed at baseline and again at 25 days post-AAV delivery. No effects were identified at baseline for both sexes combined ($F(3, 30) = 0.0708, p = 0.9751$) or for males alone ($F(3, 17) = 0.2115, p = 0.8870$). Due to the low sample size per groups no statistical analysis were performed for females alone.

At 25 days post-AAV delivery an effect of treatment was identified for both sexes combined ($F(3, 30) = 3.211, p = 0.0369$). Post-hoc comparisons identified no differences between the groups. No effects were identified for males alone ($F(3, 17) = 1.597, p = 0.2272$). Due to the low sample size per groups no statistical analysis were performed for females alone (Figure 4.9).

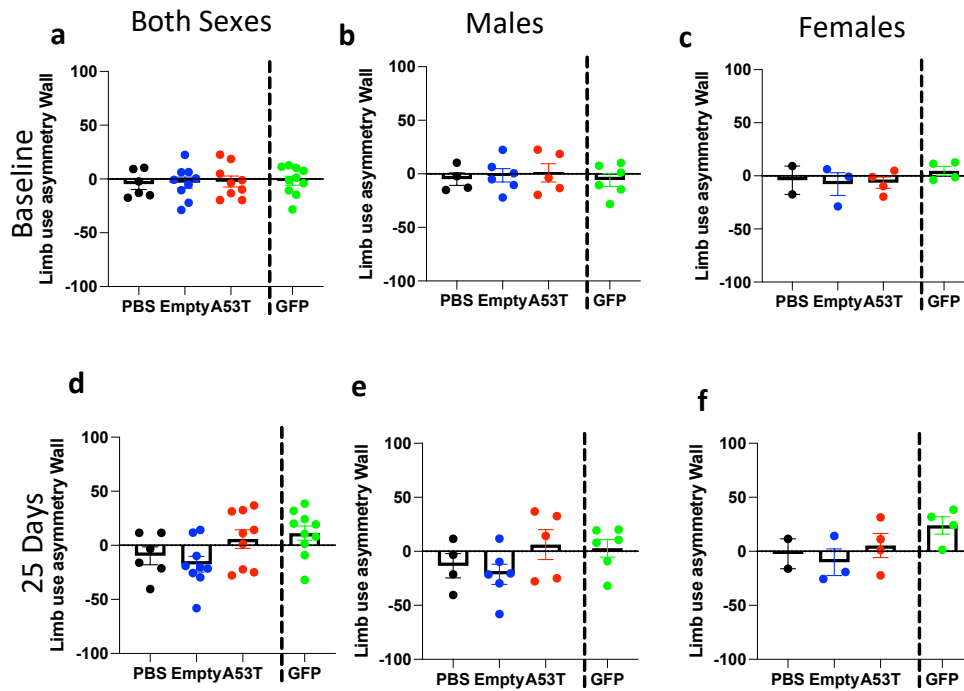


Figure 4.9: Limb use asymmetry in the cylinder test at baseline and 25 days post-AAV delivery. No limb use asymmetry was observed at baseline or 25 days post-AAV delivery. Data presented as mean with SEM. One-way ANOVA followed by Neuman-Keuls multiple comparisons test. $n = 6 - 10$ (a, d) both sexes combined, $n = 4 - 6$ (b, e) males, $n = 2 - 4$ (c, f) females.

4.4.4 Stepping Test

4.4.4.1 There were no differences at baseline between the treatment groups in the stepping test with both sexes combined, or males analysed alone.

Baseline performance in the stepping test showed no differences between the treatment groups for either both sexes combined or when males were analysed alone. Due to the low sample size per groups no statistical analysis were performed for females alone in the stepping test. Data presented in Appendix II.

4.4.4.2 There were no differences between the treatment groups in the stepping test 24 days post-AAV delivery.

Analysis of the number of forward steps 24 days post-AAV delivery found an effect of side in the forward direction (Interaction $F(3, 60) = 0.0295$, $p = 0.9931$; Side $F(1, 60) = 4.392$, $p = 0.0403$; Treatment $F(3, 60) = 2.666$, $p = 0.0588$). Post-hoc comparisons did not identify any differences between the treatment groups. Analysis of the number of backward steps identified an effect of treatment (Interaction $F(3, 60) = 0.0583$, $p = 0.9813$; Side $F(1, 60) = 2.943$, $p = 0.0914$; Treatment $F(3, 60) = 6.500$, $p = 0.0007$). Post-hoc comparisons did not identify any differences between the treatment groups.

Analysis of percentage forward steps at 24 days post-AAV delivery found an effect of side (Interaction $F(3, 60) = 0.0835$, $p = 0.9688$; Side $F(1, 60) = 11.40$, $p = 0.0013$; Treatment $F(3, 60) = 0.000$, $p > 0.9999$). Post-hoc comparisons did not identify any differences between the treatment groups. No effects were identified when percentage backward steps were analysed (Interaction $F(3, 60) = 0.3436$, $p = 0.7939$; Side $F(1, 60) = 3.761$, $p = 0.0572$; Treatment $F(3, 60) = 0.000$, $p > 0.9999$) (Figure 4.10).

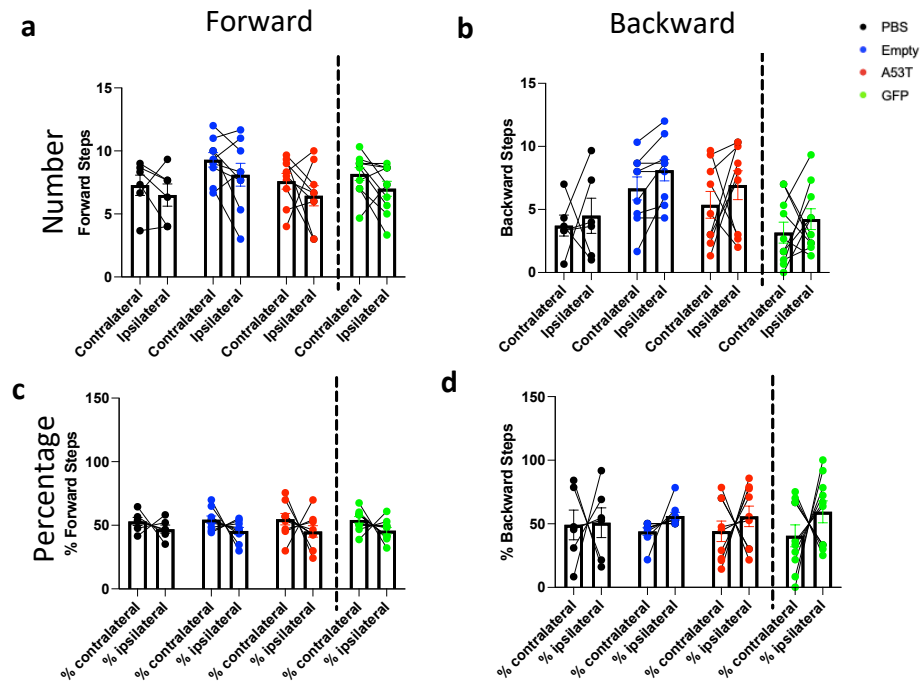


Figure 4.10: Performance in the stepping test at 24 days post-AAV delivery for both sexes combined. No differences were identified between the treatment groups in the stepping test at 24 days post-AAV delivery. Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keul's multiple comparisons test. n = 6-10.

4.4.4.3 There were no differences between the treatment groups in the stepping test 24 days post-AAV delivery when males were analysed alone.

Analysis of the number of forward steps at 24 days post-AAV delivery found an effect of side (Interaction $F(3, 34) = 0.0910$, $p = 0.9645$; Side $F(1, 34) = 7.059$, $p = 0.0119$; Treatment $F(3, 34) = 0.6876$, $p = 0.5659$). No differences were identified between the treatment groups through post-hoc comparisons. Analysis of the number of backward steps identified an effect of treatment (Interaction $F(3, 34) = 0.7641$, $p = 0.5321$; Side $F(1, 34) = 0.0425$, $p = 0.8379$; Treatment $F(3, 34) = 4.622$, $p = 0.0081$). No differences were identified between the treatment groups through post-hoc comparisons.

An effect of side was identified for percentage forward steps (Interaction $F(3, 34) = 0.1849$, $p = 0.9059$; Side $F(1, 34) = 21.03$, $p < 0.0001$; Treatment $F(3, 34) = 0.000$, $p > 0.9999$). No differences were identified between the treatment groups through post-hoc comparisons. No effects were identified when the percentage backward steps were analysed (Interaction $F(3, 34) = 1.222$, $p = 0.3167$; Side $F(1, 34) = 0.0665$, $p = 0.7981$; Treatment $F(3, 34) = 0.000$, $p > 0.9999$) (Figure 4.11).

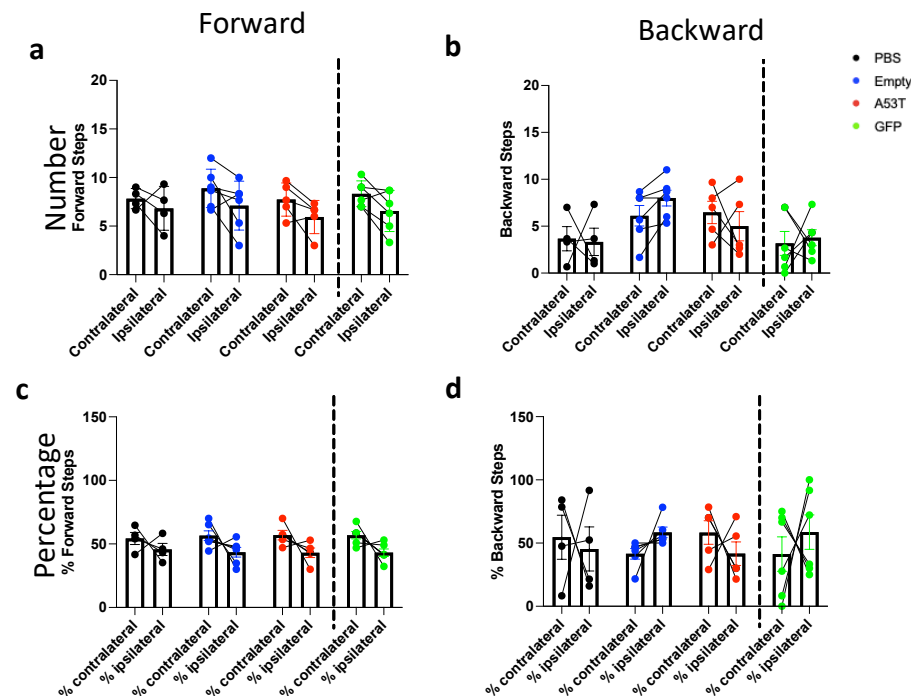


Figure 4.11: Performance in the stepping rest at 24 days post-AAV delivery for males alone. No differences were identified between the treatment groups in the stepping test at 24 days post-AAV delivery when males were analysed alone. Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 4-6.

4.4.4.4 Performance in the Stepping Test for females alone at 24 days post-AAV delivery.

Due to the low sample size per groups no statistical analysis were performed for females alone. The data are presented graphically in Figure 4.12 below.

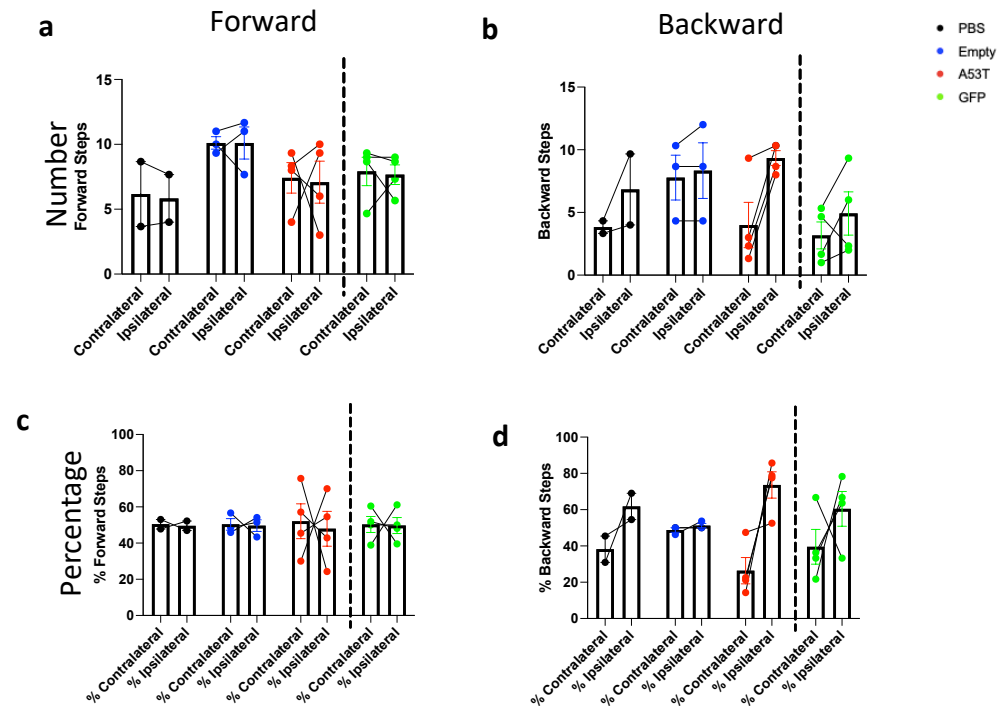


Figure 4.12: Performance in the stepping test at 24 days post-AAV delivery for females alone. Data expressed as mean with standard error of the mean. n = 2-4.

4.4.5 Staircase Test

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

Two animals from the PBS group, three from the Empty group, three from the GFP group and three from the A53T group were removed from the analysis as they met the criteria for unsuccessful acquisition of the Staircase test as outlined in Table 2.2. Baseline performance in the staircase test showed no differences between the treatment groups for either both sexes combined or when males were analysed alone. Due to the low sample size per groups no statistical analysis were performed for females alone in the staircase test. Data presented in Appendix II.

4.4.5.2 No differences were identified between the groups in the staircase test at 21-23 days post-AAV delivery.

No effects were identified with both sexes combined for number of pellets retrieved (Interaction $F(3, 38) = 0.3986$, $p = 0.7547$; Side $F(1, 38) = 2.495$, $p = 0.1225$; Treatment $F(3, 38) = 0.5410$, $p = 0.6571$). Due to the low sample size per groups no statistical analysis were performed for male or females separately in the staircase test.

Percentage success was also analysed at 21-23 days post-AAV delivery in the staircase test. No effects were identified for both sexes combined (Interaction $F(3, 38) = 0.2142$, $p = 0.8860$; Side $F(1, 38) = 0.2931$, $p = 0.5914$; Treatment $F(3, 38) = 0.6532$, $p = 0.5859$) (Figure 4.13). Due to the low sample size per groups no statistical analysis were performed for males of females separately in the staircase test.

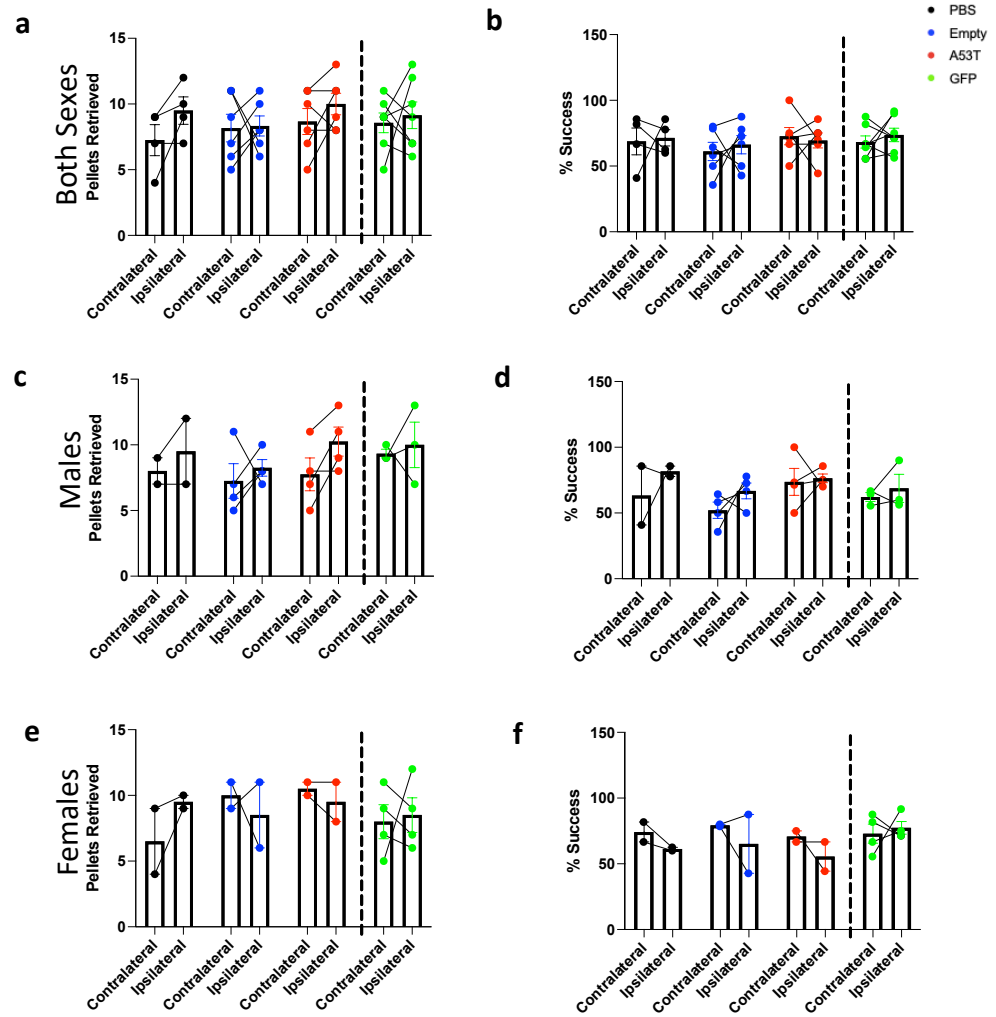


Figure 4.13: Number of pellets retrieved and percentage success at 21-23 days post-AAV delivery in the staircase test. No differences were identified between the treatment groups at 21-23 days post-AAV delivery. Data presented as mean with SEM. Two-way ANOVA. $n = 4 - 7$ (a, b) both sexes combined, $n = 2 - 5$ (c, d) males, $n = 2 - 4$ (e, f) females.

4.4.6 Tyrosine hydroxylase immunoreactive cells

4.4.6.1 Decreased TH immunoreactive cells were observed in the substantia nigra of AAV-A53T and AAV-GFP treatment groups when compared with the PBS treatment group when both sexes were combined.

The number of TH immunoreactive 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. An effect was identified when both sexes were analysed together ($H(3) = 12.13$, $p = 0.0070$), with differences identified between the AAV-A53T group and the PBS group ($p = 0.0049$), and between the AAV-GFP and the PBS group ($p = 0.0394$). No effects identified when males were analysed alone ($H(3) = 7.036$, $p = 0.0708$) (Figure 4.14). Due to the low sample size per groups no statistical analysis were performed for females alone.

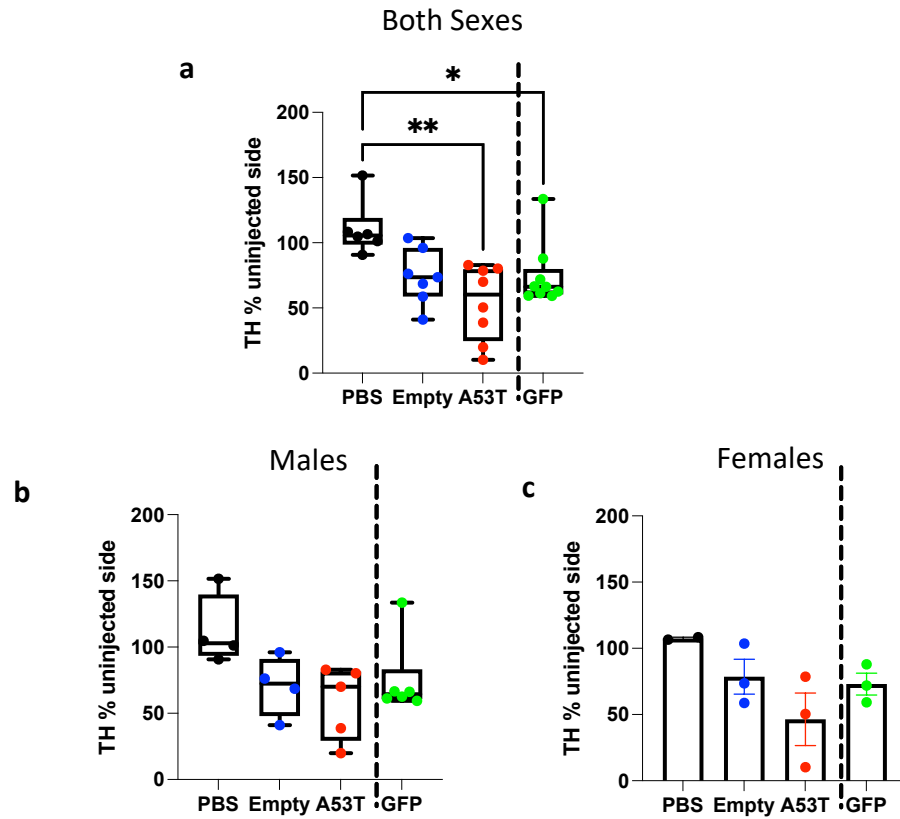


Figure 4.14: Analysis of TH immunoreactive cell counts in the substantia nigra expressed as a percentage of the un-injected side. Decreased TH immunoreactive cells were found in both the AAV-A53T and AAV-GFP treatment groups when compared with the PBS treatment group at 25 days post-AAV delivery when both sexes were combined (a). Data presented as median with minimum and maximum (a, b), or mean with SEM (c). Kruskal-Wallis test followed by Dunn's multiple comparisons test (a, b), or one-way ANOVA (c). * $p < 0.05$, ** $p < 0.01$. $n = 5 - 8$ (a) both sexes combined, $n = 4 - 5$ (b) males, $n = 2 - 3$ (c) females.

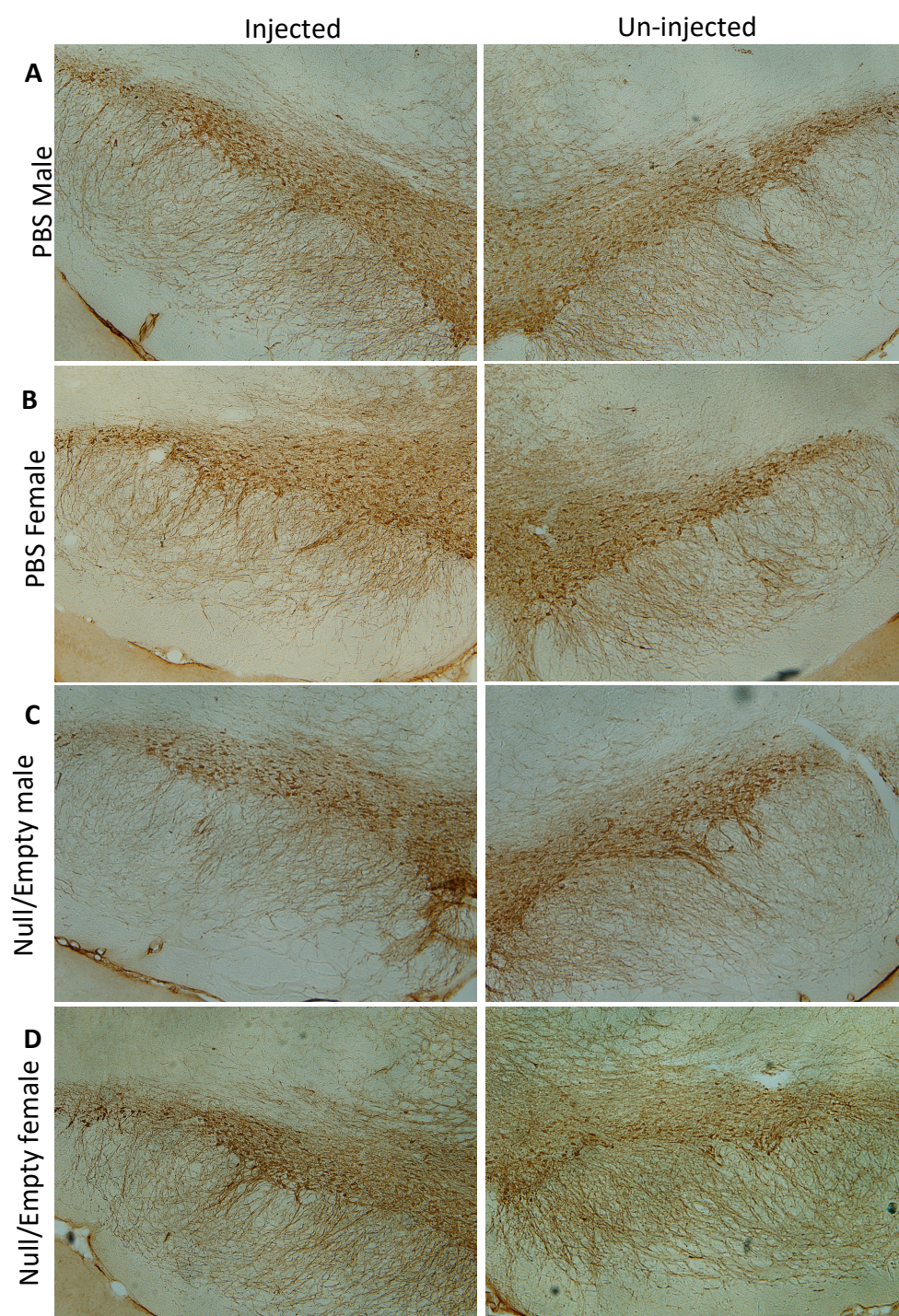


Figure 4.15: Representative images of TH immunoreactivity in the substantia nigra at 3.5 weeks post-AAV delivery in (a) PBS injected male, (b) PBS injected female, (c) AAV1/2-CMV-Null/Empty injected male, (d) AAV1/2-CMV-Null/Empty injected female. Images acquired at 10X.

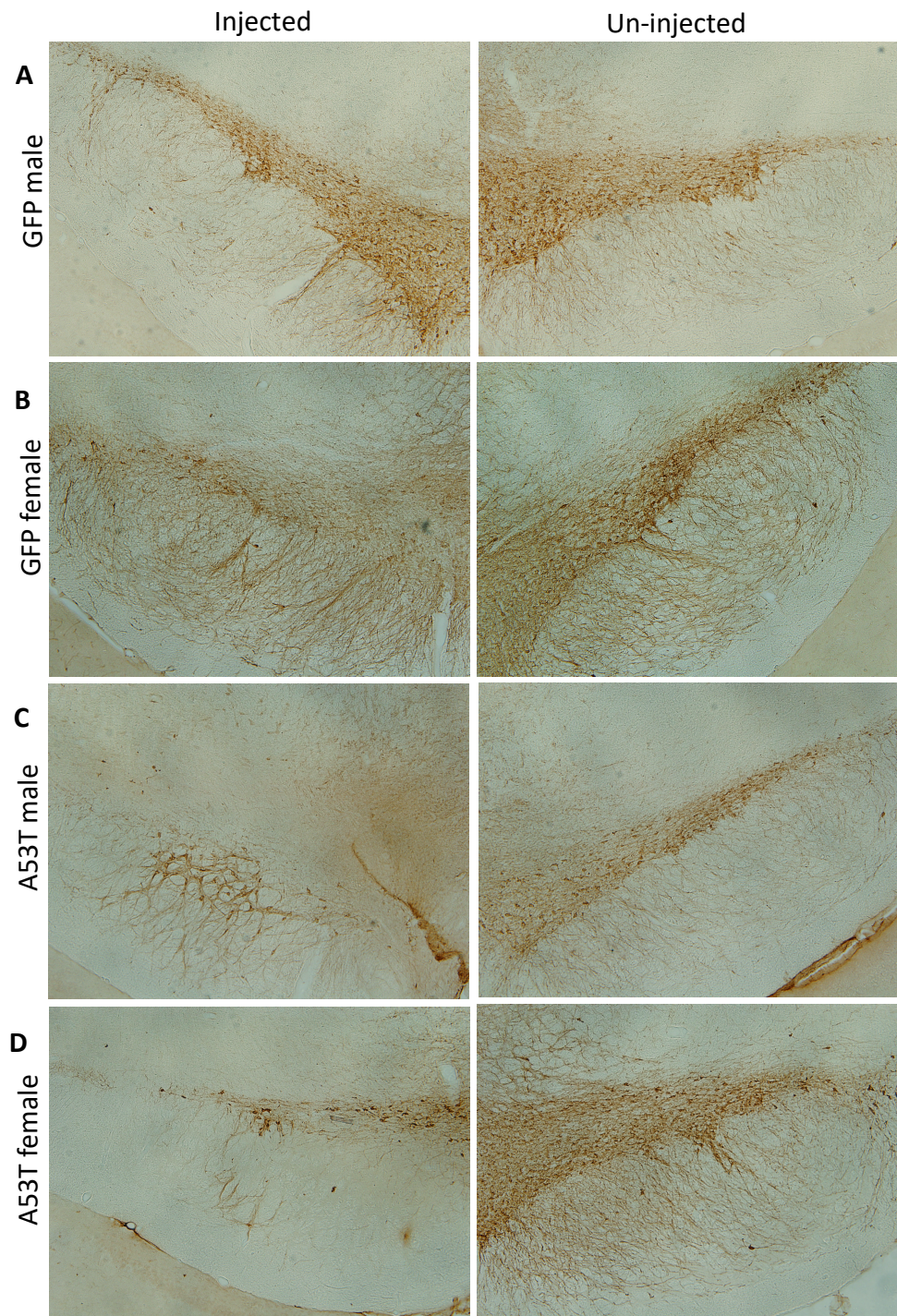


Figure 4.16: Representative images TH immunoreactivity in the substantia nigra at 3.5 weeks post-AAV delivery in (a) AAV1/2-CAG-miR scrambled control alpha synuclein co-expressing eGFP injected male, (b) AAV1/2-CAG-miR scrambled control alpha synuclein co-expressing eGFP injected female, (c) AAV1/2-CMV-A53T alpha-synuclein injected male, (d) AAV1/2-CMV-A53T alpha-synuclein injected female. Images acquired at 10X.

4.4.7 Summary of Results 3.5 week AAV-A53T Study

	Cylinder Test 25 days post-AAV delivery	Stepping Test 24 days post-AAV delivery	Staircase Test 21-23 days post- AAV delivery	TH immunoreactivity (% un-injected side) 25 days post-AAV delivery
Both Sexes	AAV-GFP decreased contralateral wall contacts when compared with PBS. AAV-GFP increased limb use asymmetry compared to AAV-empty.	No differences	No differences	Decreased in AAV- eGFP and AAV-A53T when compared with PBS.
Male	No differences	No differences	Not statistically analysed due to low number per group.	No differences
Female	Not statistically analysed due to low number per group.	Not statistically analysed due to low number per group.	Not statistically analysed due to low number per group.	Not statistically analysed due to low number per group.

Table 4.3: A summary of the results obtained in the 3.5 week AAV-A53T study.

4.5 Results – 6.5 week AAV-A53T Study

4.5.1 Alpha-synuclein immunofluorescence

4.5.1.1 Alpha-synuclein immunofluorescence intensity, expressed as arbitrary units, was found to be increased in the AAV-A53T alpha-synuclein injected substantia nigra when compared with the un-injected hemisphere at 6.5 weeks post-AAV delivery.

Comparison of the injected and un-injected hemispheres of the substantia nigra of the animals in the AAV-A53T groups found a difference between the sides with both sexes combined ($W = 55.00$, $p = 0.0020$), with no differences identified for males alone ($t(4) = 2.390$, $p = 0.0752$) or for females alone ($W = 15.00$, $p = 0.0625$) (Figure 4.17).

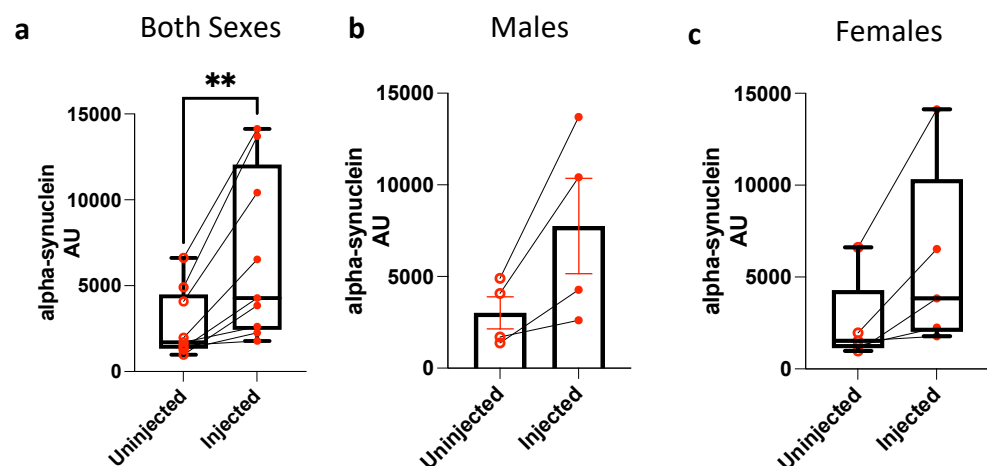


Figure 4.17: Comparison of immunofluorescence intensity in the injected and un-injected hemispheres of the substantia nigra of the animals in the AAV-A53T group at 6.5 weeks post-AAV delivery. Increased alpha-synuclein immunofluorescence intensity was observed in the injected hemisphere when compared to the un-injected hemisphere with both sexes combined (a). Data presented as mean with SEM (b) or median with minimum to maximum (a, c). Paired t-test or Wilcoxon matched-pairs signed rank test. AU = arbitrary units. $n = 9$ (a) both sexes combined, $n = 4$ (b) males, $n = 5$ (c) females. ** $p < 0.01$.

4.5.1.2 Alpha-synuclein immunofluorescence intensity in the substantia nigra, expressed as a percentage of the un-injected side, was found to be increased in the AAV-A53T alpha-synuclein injected animals when compared with the AAV-empty injected animals at 6.5 weeks post-AAV delivery.

Analysis of alpha-synuclein immunofluorescence in the substantia nigra as a percentage of the un-injected side in the AAV-empty and AAV-A53T groups found a difference between the groups when both sexes were combined ($t(9.852) = 4.304$, $p = 0.0016$) and when males were analysed ($t(4.644) = 3.074$, $p = 0.0305$) (Figure 4.18). Due to the low sample size per groups no statistical analysis were performed for females alone.

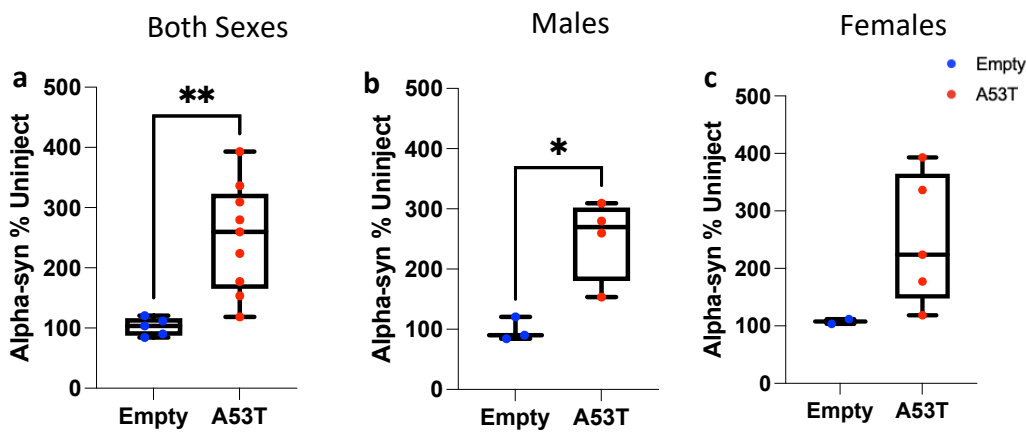


Figure 4.18: Comparison of the immunofluorescence intensity in the substantia nigra of alpha-synuclein when expressed as a percentage of the un-injected side for the AAV-empty and AAV-A53T groups at 6.5 weeks post-AAV delivery. Increased alpha-synuclein immunofluorescence, analysed as a percentage of the un-injected side, was found in the AAV-A53T group when compared to the AAV-empty groups. Data presented as median with minimum and maximum. Unpaired t-test with Welch's correction. $n = 5-9$ (a) both sexes combined, $n = 3-4$ (b) males, $n = 2-5$ (c) females. * $p < 0.05$, ** $p < 0.01$.

4.5.1.3 Review of the AAV-A53T alpha-synuclein injected animals found varying immunofluorescence across the substantia nigra and between animals at 6.5 weeks post-AAV delivery.

Alpha-synuclein immunofluorescence was assessed in the brains of AAV-A53T treated animals and a sample of the AAV-empty treated animals in both the injected and un-injected hemispheres. A number of sections, each 175µm apart, were analysed for immunofluorescence intensity of alpha-synuclein, as outlined in Table 4.4. Data were assessed as the alpha-synuclein immunofluorescence in the un-injected side divided by the alpha-synuclein immunofluorescence in the injected side. Due to the serial nature of the tissue sectioning and immunofluorescence staining on a series of sections, it is possible the exact location where the AAV was delivered was not assessed in every animal.

Animal	Sex	Treatment Group	Expression over 100% (number of sections)
1	Female	A53T	2/2
2	Male	A53T	2/2
4	Male	A53T	2/2
5	Female	A53T	2/2
6	Male	A53T	1/1
8	Female	A53T	2/2
10	Male	A53T	2/2
14	Female	A53T	2/2
16	Male	A53T	2/2
19	Female	A53T	2/2
3	Male	Empty	2/2
7	Male	Empty	0/2
12	Male	Empty	0/2
17	Female	Empty	1/1
18	Female	Empty	1/2

Table 4.4: Analysis of alpha-synuclein immunofluorescence in the substantia nigra in the injected and un-injected hemisphere of animals in the AAV-A53T alpha-synuclein and AAV-empty groups. Expression over 100% represents alpha-synuclein immunofluorescence in the injected hemisphere expressed as percentage of the un-injected side, that is greater than 100%. The data represented in this table are taken from a number of sections from the substantia nigra.

Representative images of alpha-synuclein and TH positive immunofluorescence in the substantia nigra of male and female AAV1/2-CMV-A53T alpha-synuclein treated animals (Figure 4.19).

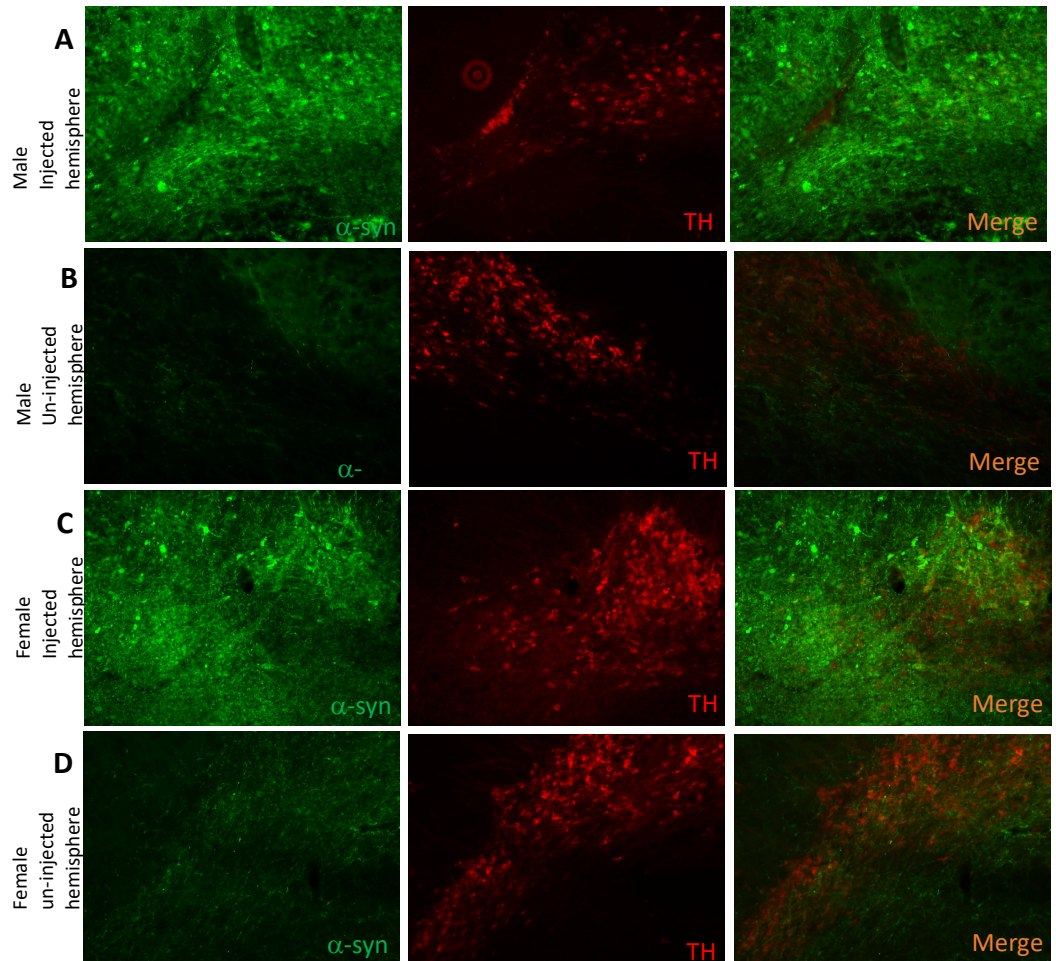


Figure 4.19: Representative images of alpha-synuclein and TH immunofluorescence in the substantia nigra 6.5 weeks post-AAV delivery. (A) A53T alpha-synuclein injected male injected hemisphere, (B) A53T alpha-synuclein injected male un-injected hemisphere, (C) A53T alpha-synuclein injected female injected hemisphere, (D) A53T alpha-synuclein injected female un-injected hemisphere. First column represents alpha-synuclein immunofluorescence, second column represents TH immunofluorescence, third column represents merged images of the first 2 columns. Images acquired at 20X.

4.5.1.4 One animal in the AAV-A53T group was identified as expressing alpha-synuclein immunofluorescence at a level comparable to the AAV-empty injected group.

Reviewing the immunofluorescence of alpha-synuclein in the AAV-A53T group as a percentage of the un-injected side found no animals expressing alpha-synuclein immunofluorescence in the injected hemisphere less than 100% of the un-injected hemisphere. Due to the inclusion of the AAV-empty group in the alpha-synuclein immunofluorescence analysis, the mean, standard deviations and SEM when the data were presented as percentage of the un-injected hemisphere was calculated for both the AAV-A53T and AAV-empty groups.

Group	Number of Values	Minimum	Maximum	Range	Mean	Standard Deviation	SEM
Empty	5	84.22	120.7	36.52	102.1	15.05	6.730
A53T	10	108.9	393.0	284.2	236.1	96.07	30.38

Table 4.5: Descriptive statistics for alpha-synuclein expression as a percentage of the un-injected side in the AAV-empty and AAV-A53T groups in the substantia nigra.

Reviewing the data, there was one animal in the AAV-A53T group identified whose alpha-synuclein immunofluorescence expressed as percentage un-injected side fell within the mean $\pm$ the standard deviation of the AAV-empty group. It was decided that this animal be removed from behaviour data analysis as this animal had immunofluorescence levels of alpha-synuclein comparable to animals within the AAV-empty group.

4.5.2 Staircase Test

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

One animal from the Empty group and two animals from the A53T group were removed from the analysis as they met the criteria for unsuccessful acquisition of the Staircase test as outlined in the Table 2.2 No differences were identified at baseline between the treatment groups at baseline when both sexes were analysed in combination, or when males and females were analysed separately. Data presented in Appendix II.

4.5.2.2 No differences were identified between the groups in the staircase test at 23 days post-AAV delivery.

No effects were identified with both sexes combined for number of pellets retrieved (Interaction $F(1, 30) = 0.2027, p = 0.6558$; Side $F(1, 30) = 0.0133, p = 0.9091$; Treatment $F(1, 30) = 0.4922, p = 0.4883$), for males alone (Interaction $F(1, 10) = 0.0000, p > 0.9999$; Side $F(1, 10) = 0.6489, p = 0.4392$; Treatment $F(1, 10) = 0.5453, p = 0.4772$), or for females alone (Interaction $F(1, 16) = 0.5714, p = 0.4607$, Side $F(1, 16) = 0.5714, p = 0.4607$; Treatment $F(1, 16) = 0.0635, p = 0.8043$) in the staircase test at 23 days post-AAV delivery.

The percentage success was analysed at 23 days post-AAV delivery in the staircase test. No effects were identified when male and female were analysed together (Interaction $F(1, 30) = 0.0153, p = 0.9024$; Side $F(1, 30) = 0.1672, p = 0.6855$; Treatment $F(1, 30) = 0.0536, p = 0.8185$), when males were analysed alone (Interaction $F(1, 10) = 0.1452, p = 0.7112$; Side $F(1, 10) = 0.1532, p = 0.7037$; Treatment $F(1, 10) = 0.031, p = 0.8638$), or when females were analysed alone (Interaction $F(1, 16) = 0.3789, p = 0.5468$; Side $F(1, 16) = 2.694, p = 0.1202$; Treatment $F(1, 16) = 0.4470, p = 0.5133$) (Figure 4.20).

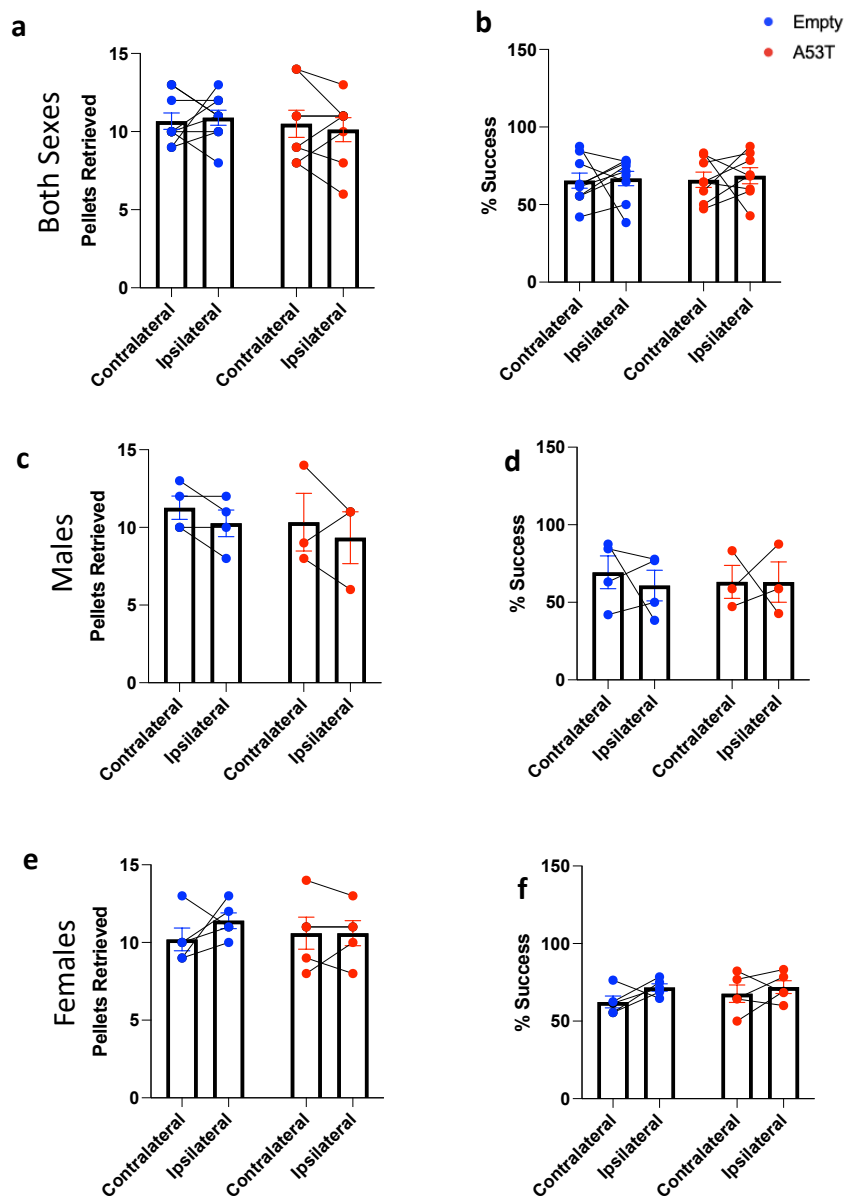


Figure 4.20: Number of pellets retrieved and percentage success at 23 days post-AAV delivery in the staircase test. No effects were identified for either number of pellets retrieved or percentage success. Data presented as mean with SEM. Two-Way ANOVA. $n = 7 - 9$ (a, b) both sexes combined, $n = 3 - 4$ (c, d) males, $n = 4 - 5$ (e, f) females.

4.5.2.3 No differences were identified between the groups in the staircase test at 43 days post-AAV delivery.

The staircase test was repeated at 43 days post-AAV delivery. An effect of side was identified for number of pellets retrieved when both sexes were combined (Interaction $F(1, 30) = 0.0441, p = 0.8351$; Side $F(1, 30) = 5.083, p = 0.0316$; Treatment $F(1, 30) = 0.0441, p = 0.8351$). Post-hoc comparisons did not identify any differences between the treatment groups. No effects were identified when males were analysed alone (Interaction $F(1, 10) = 0.020, p = 0.8904$; Side $F(1, 10) = 1.095, p = 0.3200$; Treatment $F(1, 10) = 2.420, p = 0.1509$). Analysis of females alone found an effect of side (Interaction $F(1, 16) = 0.3333, p = 0.5717$; Side $F(1, 16) = 6.750, p = 0.0194$; Treatment $F(1, 16) = 4.083, p = 0.0604$). Post-hoc comparisons did not identify any differences between the treatment groups.

No effects were identified for the success rate when male and female were analysed together (Interaction $F(1, 30) = 2.641, p = 0.1146$; Side $F(1, 30) = 1.700, p = 0.2022$; Treatment $F(1, 30) = 0.0032, p = 0.9547$), or when females were analysed alone (Interaction $F(1, 16) = 0.4566, p = 0.5089$; Side $F(1, 16) = 2.463, p = 0.1361$; Treatment $F(1, 16) = 1.194, p = 0.2908$). When males were analysed alone an interaction effect was identified (Interaction $F(1, 10) = 4.996, p = 0.0494$; Side $F(1, 10) = 0.0129, p = 0.9118$; Treatment $F(1, 10) = 2.740, p = 0.1228$). Post-hoc comparisons did not identify any differences between the treatment groups (Figure 4.21).

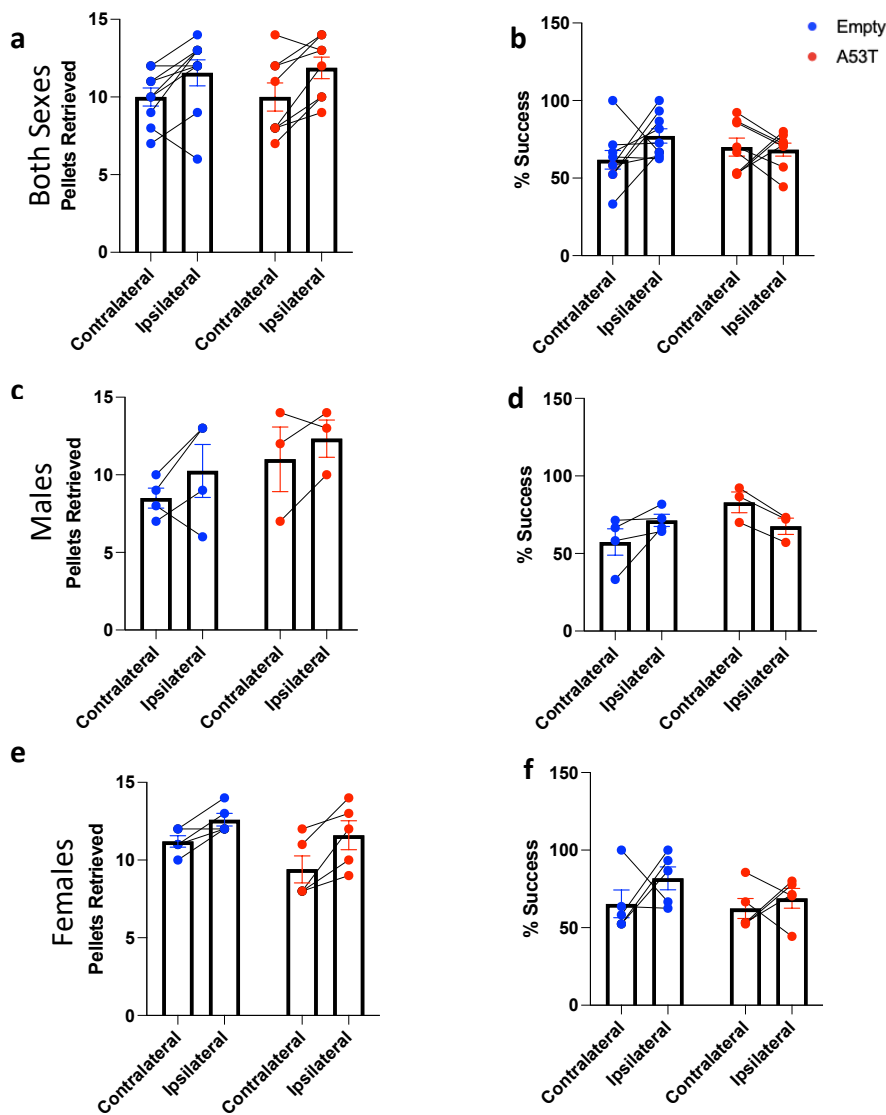


Figure 4.21: Number of pellets retrieved and percentage success rate at 43 days post-AAV delivery in the staircase test. No differences were identified between the treatment groups at 43 days post-AAV delivery in the staircase test. Data presented as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. $n = 7 - 9$ (a, b) both sexes, $n = 3 - 4$ (c, d) males, $n = 4 - 5$ (e, f) females.

4.5.3 Cylinder Test

4.5.3.1 No limb use asymmetry was identified in the cylinder test when both sexes were analysed together at 44 days post-AAV delivery.

No differences between the groups were identified at 44 days post-AAV delivery when both sexes were combined for number of contralateral wall contacts ($t(17) = 0.6202$, $p = 0.5433$), percentage contralateral wall contacts ($t(17)=0.0096$, $p = 0.9925$), number of ipsilateral wall contacts ($t(17)=0.2934$, $p = 0.7728$), percentage ipsilateral wall contacts ($t(17)= 1.261$, $p = 0.2243$), number of simultaneous wall contacts ($U = 30.50$, $p = 0.2509$), or percentage simultaneous wall contacts ($t(18) = 1.571$, $p = 0.1336$) (Figure 4.22).

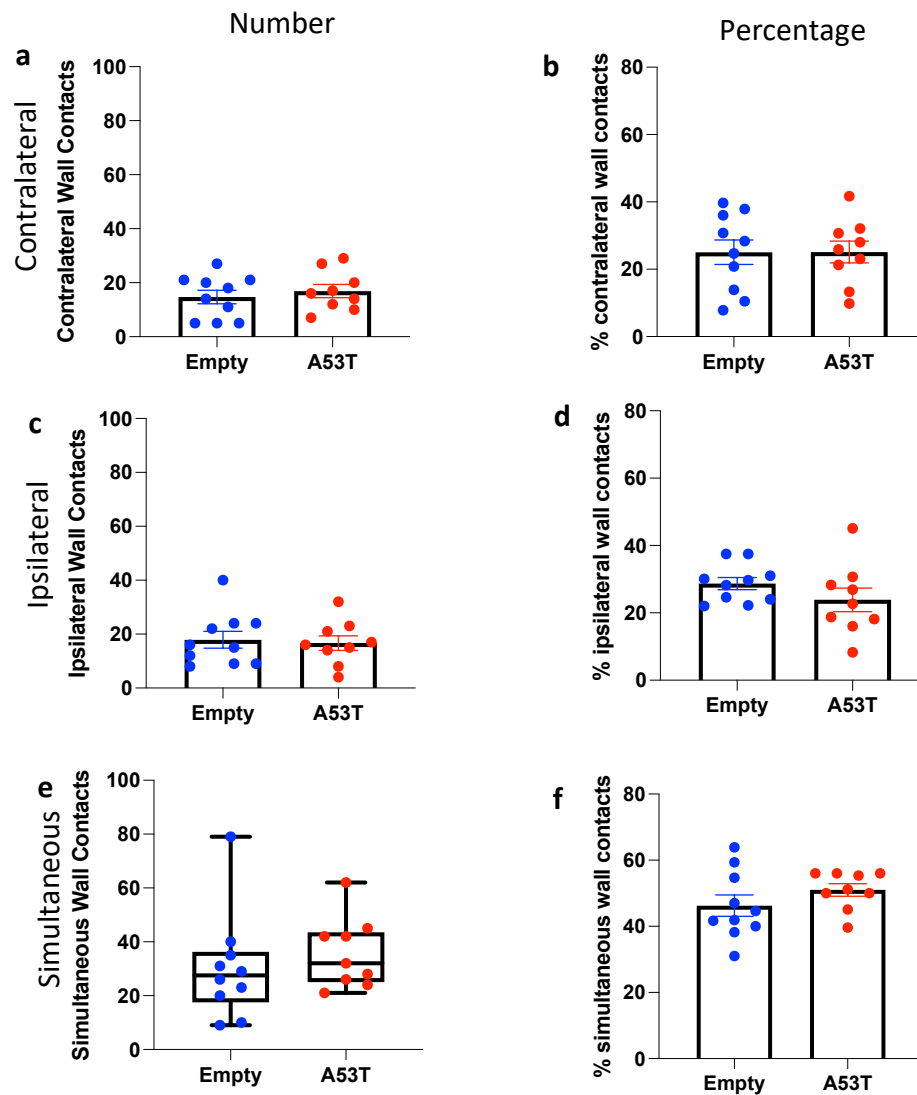


Figure 4.22: Performance in the cylinder test at 44 days post-AAV delivery. No differences were identified between the treatment groups in the cylinder test at 44 days post-AAV delivery when both sexes were combined. Data presented as mean with SEM (a, b, c, d, f), or median with minimum and maximum (e). Unpaired t-test or Mann Whitney test. n = 9 – 10.

4.5.3.2 No limb use asymmetry was identified in the cylinder test when males were analysed alone at 44 days post-AAV delivery.

Analysis of performance in the cylinder test at 44 days post-AAV delivery found no differences for males for number of contralateral wall contacts ($t(7) = 0.7264$, $p = 0.4912$), percentage contralateral wall contacts ($t(7)=0.1576$, $p = 0.8792$), number of ipsilateral wall contacts ($t(7)=0.1318$, $p = 0.8988$), percentage ipsilateral wall contacts ($t(7) = 1.674$, $p = 0.1381$), number of simultaneous wall contacts ($t(7)=1.975$, $p = 0.0889$) or percentage simultaneous wall contacts ($t(7)=1.043$, $p = 0.3317$) (Figure 4.23).

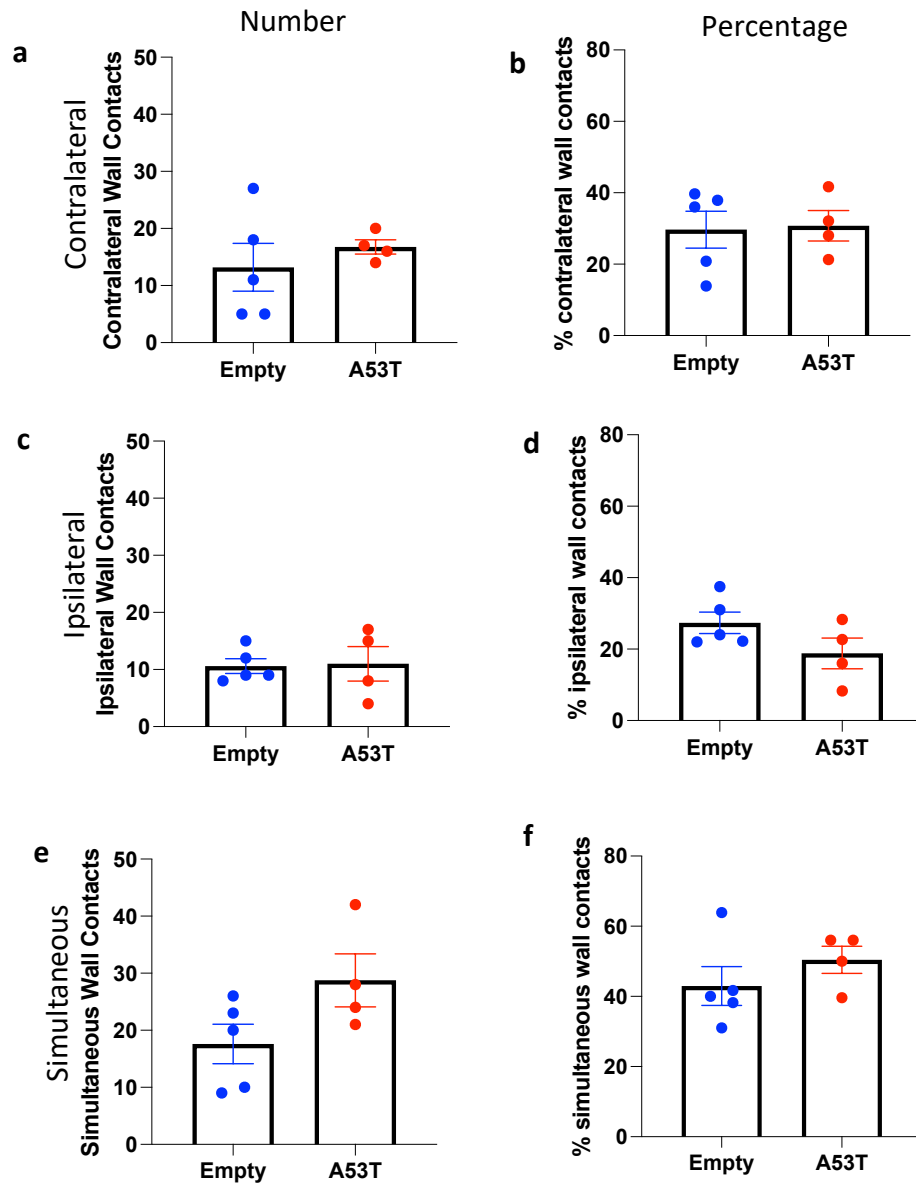


Figure 4.23: Performance in the cylinder test 44 days post-AAV delivery for males alone. No differences were identified between the treatment groups in the cylinder test at 44 days post-AAV delivery when males were analysed alone. Data presented as mean with SEM. Unpaired t-test. n = 4-5.

4.5.3.3 No limb use asymmetry was identified in the cylinder test when females were analysed alone at 44 days post-AAV delivery.

Analysis of performance in the cylinder test at 44 days post-AAV delivery found no differences for females for the number of contralateral wall contacts ($t(8) = 0.1450$, $p = 0.8883$), percentage contralateral wall contacts ($t(8)=0.0213$, $p = 0.9835$), number of ipsilateral wall contacts ($t(8)=0.7878$, $p = 0.4535$), percentage ipsilateral wall contacts ($t(8) = 0.3957$, $p = 0.7026$), number of simultaneous wall contacts ($U = 11$, $p = 0.8413$), or percentage simultaneous wall contacts ($t(8)= 0.5193$, $p = 0.6176$) (Figure 4.24).

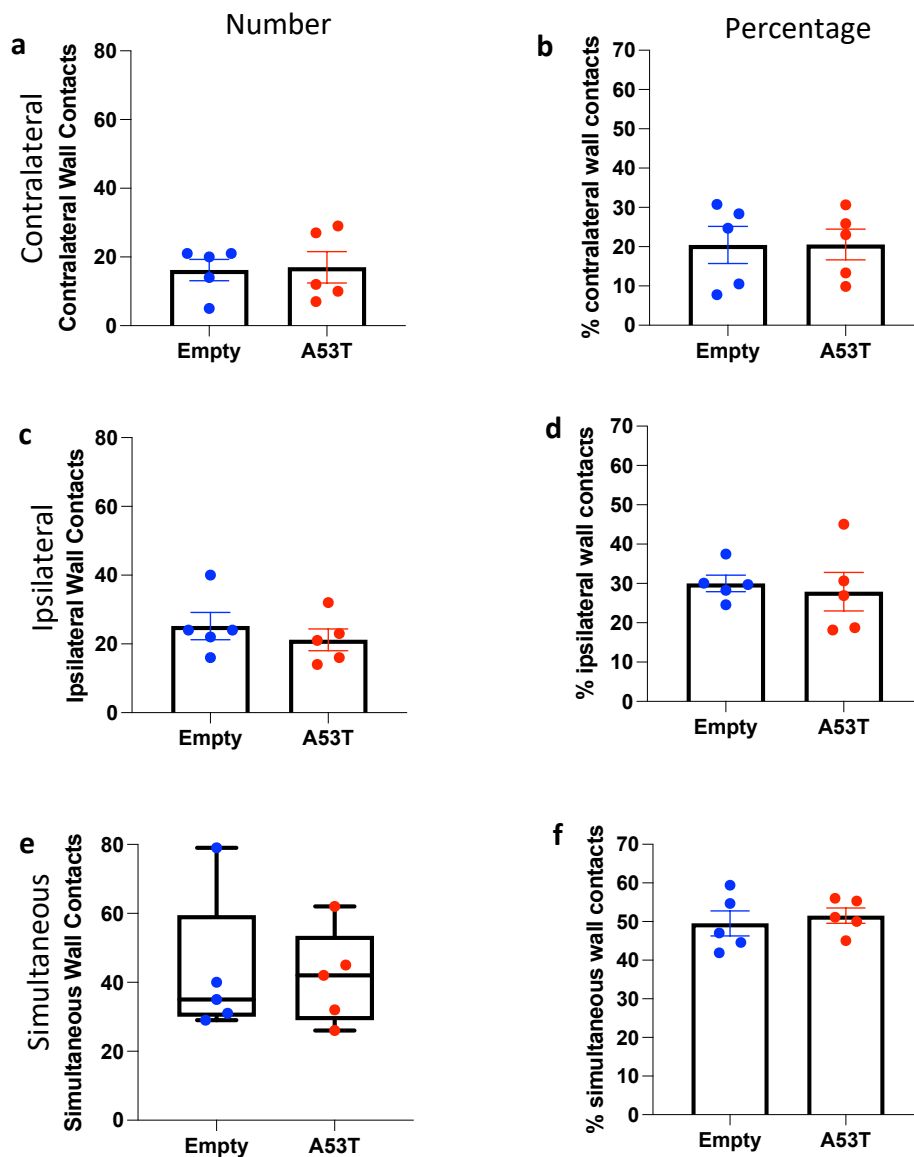


Figure 4.24: Performance in the cylinder test 44 days post-AAV delivery for females alone. No differences were identified between the treatment groups in the cylinder test at 44 days post-AAV delivery. Data presented as mean with SEM (a, b, c, d, f) or median with minimum and maximum (e). Unpaired t-test (a, b, c, d, f) or Mann Whitney test (e). $n = 5$.

4.5.3.4 No limb use asymmetry was identified in the cylinder test as assessed through the limb use asymmetry score at 44 days post-AAV delivery.

No differences were identified at 44 days post-AAV delivery in limb use asymmetry for both sexes combined ($t(17) = 0.6144$, $p = 0.5471$), males alone ($t(7) = 0.9734$, $p = 0.3628$), or females alone ($t(8) = 0.2070$, $p = 0.8412$).

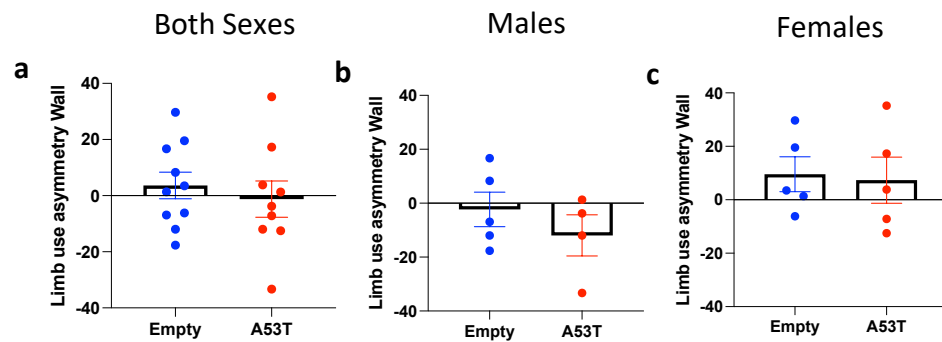


Figure 4.25: Limb use asymmetry 44 days post-AAV delivery in the cylinder test. Analysis of limb use asymmetry 44 days post-AAV delivery found no differences between the treatment groups. Data presented as mean with SEM. Unpaired t-test. $n = 9-10$ (a) both sexes combined, $n = 4-5$ (b) males, $n = 5$ (c) females.

4.5.4 Stepping Test

4.5.4.1 There were no differences at baseline between the treatment groups in the stepping test when both sexes were combined and when males were analysed alone.

No differences were identified at baseline in the stepping test when both sexes were analysed together and when males were analysed separately. Data presented in Appendix II.

4.5.4.2 Analysis of females alone at baseline identified differences in contralateral and ipsilateral backward steps in the AAV-A53T females when compared with the AAV-empty females in the stepping test.

Decreased contralateral and ipsilateral backward steps were found in the AAV-A53T treatment group at baseline when females were analysed alone and compared to AAV-empty. This is unexpected as there should be no behavioural differences observed at baseline. However, as the contralateral and ipsilateral backward steps within the AAV-A53T treatment group did not differ from one another, this was not a concern as all comparisons will be made within group for the stepping test. Data presented in Appendix II.

4.5.4.3 A difference between ipsilateral and contralateral was identified in the AAV-A53T group when the data for backward direction were analysed as both number of steps and percentage steps at 24 days post-AAV delivery.

No effects were identified at 24 days post-AAV delivery for number of forward steps in the stepping test (Interaction $F(1, 34) = 0.3563$, $p = 0.5545$; Side $F(1, 34) = 0.4876$, $p = 0.4897$; Treatment $F(1, 34) = 1.399$, $p = 0.2452$). An effect of side was identified for number of backward steps (Interaction $F(1, 34) = 2.346$, $p = 0.1348$; Side $F(1, 34) = 15.75$, $p = 0.0004$; Treatment $F(1, 34) = 1.758$, $p = 0.1937$). Post-hoc comparisons identified a difference between the number of ipsilateral and contralateral steps for the AAV-A53T group ($p < 0.01$).

Analysis of percentage forward steps did not find any effects (Interaction $F(1, 34) = 0.6397$; $p = 0.4294$; Side $F(1, 34) = 0.5114$, $p = 0.4794$; Treatment $F(1, 34)$

= 0.000, $p > 0.9999$). Analysis of percentage backward steps identified an interaction effect and an effect of side (Interaction $F(1, 34) = 14.56$, $p = 0.0005$; Side $F(1, 34) = 80.22$, $p < 0.0001$; Treatment $F(1, 34) = 0.0000$, $p > 0.9999$). Post-hoc comparisons identified a difference between ipsilateral and contralateral for both the AAV-empty ($p < 0.001$) and AAV-A53T ($p < 0.0001$) groups (Figure 4.26).

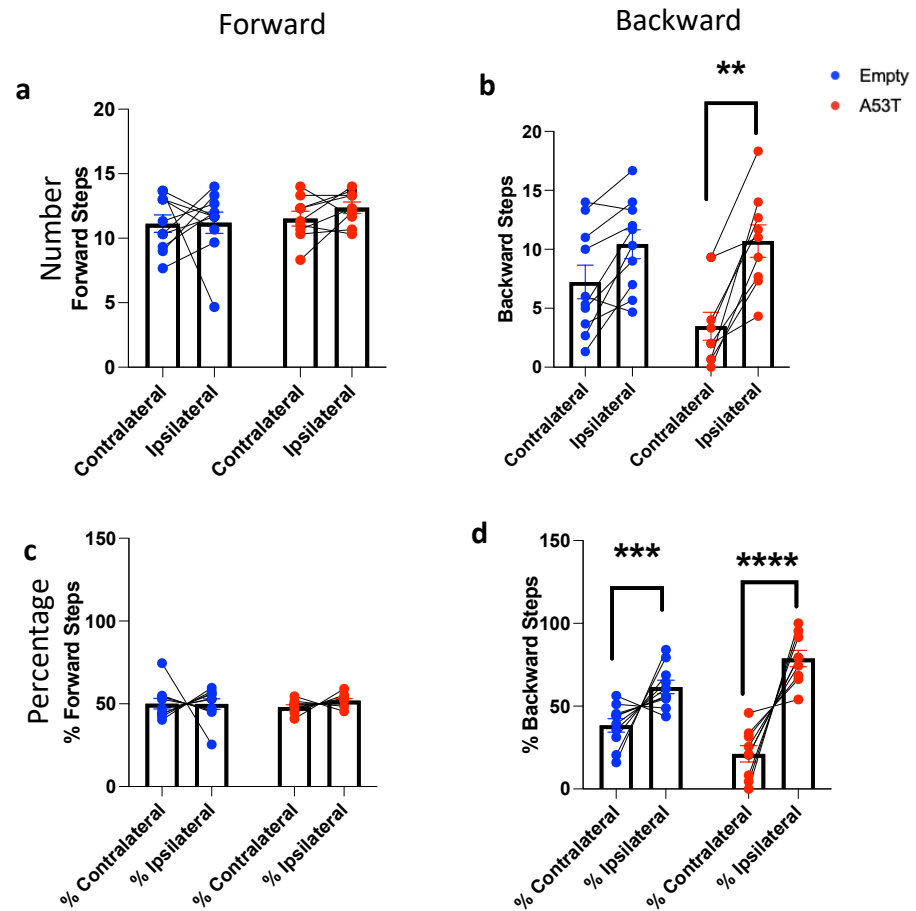


Figure 4.26: Performance in the stepping test at 24 days post-AAV delivery for both sexes combined. Decreased contralateral backward steps were identified in the AAV-A53T group when compared with the number of ipsilateral backward steps. (b) Decreased % contralateral backward steps were identified in both the AAV-empty and AAV-A53T groups when compared with their respective % ipsilateral backward steps. Data expressed mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. $n = 9-10$. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.5.4.4 A difference between ipsilateral and contralateral was identified in both the AAV-A53T and AAV-empty groups when the data for the percentage backward steps were analysed 24 days post-AAV delivery for males alone.

No effects were identified 24 days post-AAV delivery for males alone for number of forward steps (Interaction $F(1, 14) = 1.725, p = 0.2102$; Side $F(1, 14) = 0.0005, p = 0.9822$; Treatment $F(1, 14) = 0.0254, p = 0.8756$). An effect of side was identified for number of backward steps (Interaction $F(1, 14) = 1.048, p = 0.3233$; Side $F(1, 14) = 4.732, p = 0.0472$; Treatment $F(1, 14) = 0.6918, p = 0.4195$). Post-hoc comparisons did not identify any differences between the treatment groups.

No effects were identified for percentage forward steps (Interaction $F(1, 14) = 2.552, p = 0.1325$; Side $F(1, 14) = 0.0354, p = 0.8534$; Treatment $F(1, 14) = 0.000, p > 0.9999$). An effect of side was identified for percentage backward steps (Interaction $F(1, 14) = 2.306, p = 0.1511$; Side $F(1, 14) = 19.70, p = 0.0006$; Treatment $F(1, 14) = 0.000, p > 0.9999$). Post-hoc comparisons identified a difference between % ipsilateral and % contralateral in both the AAV-A53T ($p < 0.01$) and AAV-empty ($p < 0.05$) groups (Figure 4.27).

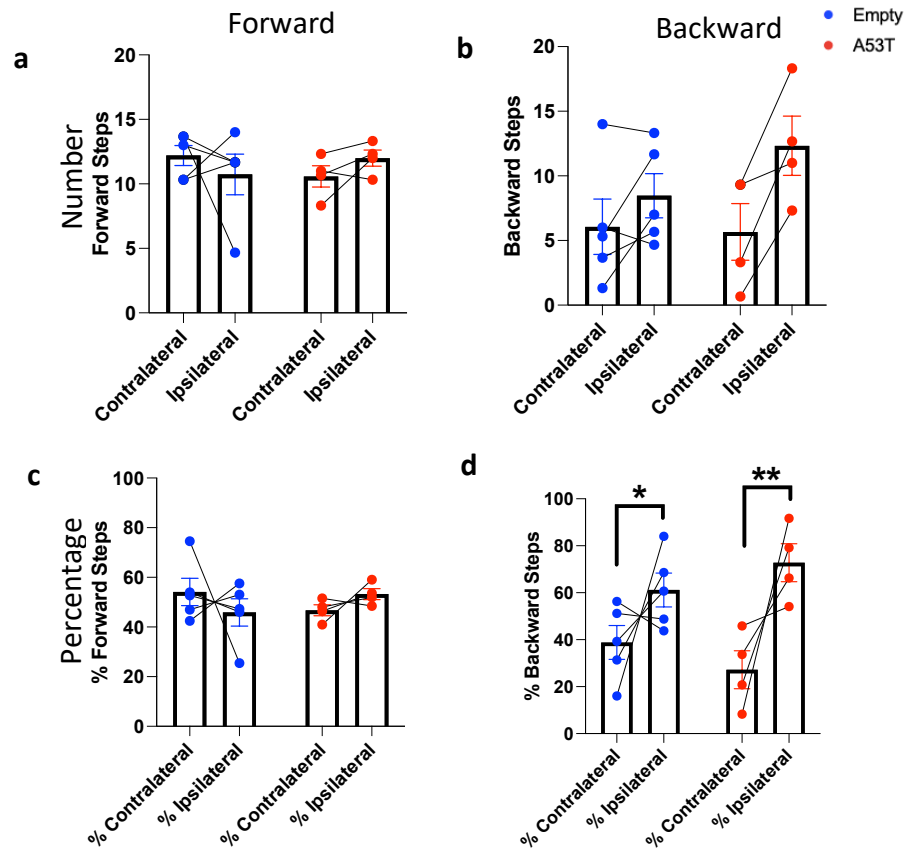


Figure 4.27: Performance in the stepping test 24 days post-AAV delivery for males alone. (d) Decreased % contralateral backward steps were identified in both the AAV-empty and AAV-A53T groups when compared with their respective % ipsilateral backward steps for males alone. Data expressed mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. $n = 4-5$. * $p < 0.05$, ** $p < 0.01$.

4.5.4.5 Decreased contralateral steps were identified in the backward direction in the AAV-A53T treatment group when females were analysed alone at 24 days post-AAV delivery.

An effect of treatment was identified for the number of forward steps for females alone at 24 days post-AAV delivery (Interaction $F(1, 16) = 0.6619$, $p = 0.4278$; Side $F(1, 16) = 1.839$, $p = 0.1939$; Treatment $F(1, 16) = 4.707$, $p = 0.0454$). Post-hoc comparisons did not identify any differences between the treatment groups. Analysis of the number of backward steps identified an effect of side and an effect of treatment when females were analysed alone (Interaction $F(1, 16) = 1.500$, $p = 0.2384$; Side $F(1, 16) = 15.19$, $p = 0.0013$; Treatment $F(1, 16) = 10.43$, $p = 0.0052$). Post-hoc comparisons identified a difference between ipsilateral and contralateral in the AAV-A53T group ($p < 0.01$).

An effect of side was identified when percentage forward steps were analysed (Interaction $F(1, 16) = 2.206$, $p = 0.1569$; Side $F(1, 16) = 5.137$, $p = 0.0376$; Treatment $F(1, 16) = 0.000$, $p > 0.9999$). Post-hoc comparisons did not identify any differences between the treatment groups. An interaction effect and an effect of side were identified for percentage backward steps (Interaction $F(1, 16) = 15.67$, $p = 0.0011$; Side $F(1, 16) = 69.85$, $p < 0.0001$; Treatment $F(1, 16) = 0.000$, $p > 0.9999$). Post-hoc comparisons identified a difference between ipsilateral and contralateral in the AAV-A53T group ($p < 0.0001$) (Figure 4.28).

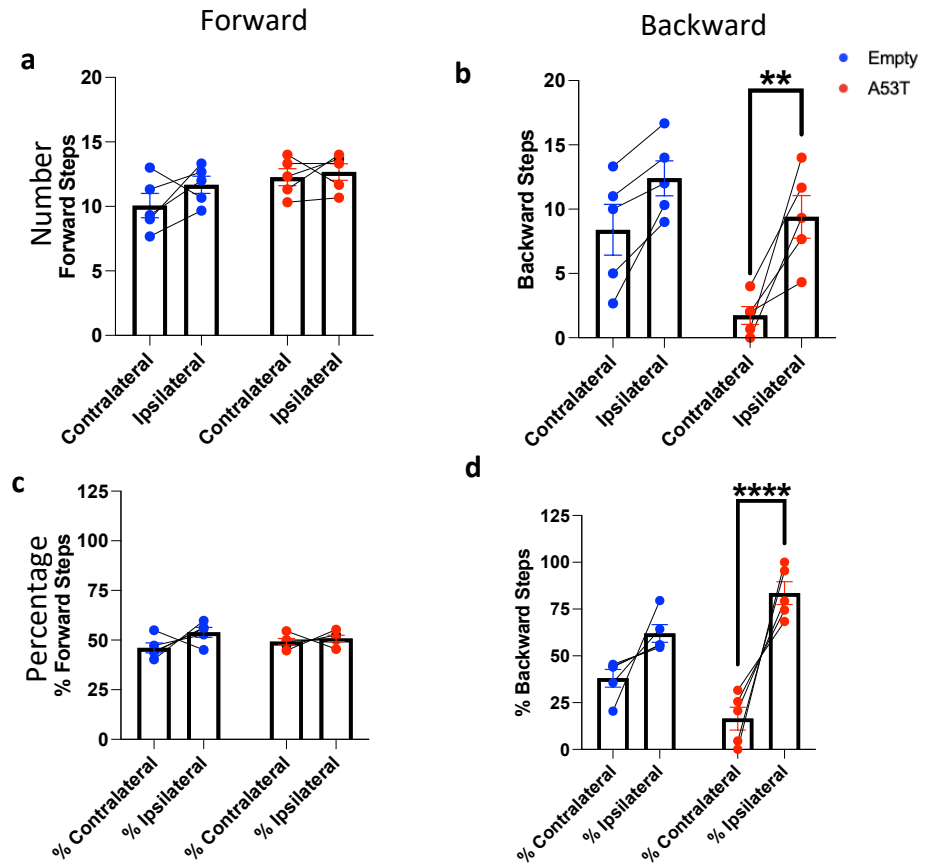


Figure 4.28: Performance in the stepping test 24 days post-AAV delivery for females alone. (b) Decreased contralateral backward steps were identified in the AAV-A53T group when compared to their ipsilateral backward steps for females alone. (d) Decreased percentage backward steps were also identified in the AAV-A53T group when compared to their percentage ipsilateral backward steps for females alone. Data expressed mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. $n = 5$. ** $p < 0.01$, **** $p < 0.0001$.

4.5.4.6 Decreased contralateral steps were identified in the backward direction in the AAV-A53T treatment group when both sexes were analysed together 42 days post-AAV delivery.

At 42 days post-AAV delivery, an effect of treatment was identified for number of forward steps (Interaction $F(1, 34) = 0.3139$, $p = 0.5790$; Side $F(1, 34) = 0.005$, $p = 0.9446$; Treatment $F(1, 34) = 7.232$, $p = 0.0110$). Post-hoc analysis found no differences between the treatment groups. Analysis of the number of backward steps identified an effect of side (Interaction $F(1, 34) = 0.4182$, $p = 0.5222$; Side $F(1, 34) = 9.909$, $p = 0.0034$; Treatment $F(1, 34) = 2.006$, $p = 0.1658$). Post-hoc comparisons identified a difference between ipsilateral and contralateral in the AAV-A53T group ($p < 0.05$).

Analysis of percentage forward steps identified no effects at 42 days post-AAV delivery (Interaction $F(1, 34) = 0.1853$, $p = 0.6696$; Side $F(1, 34) = 0.0011$, $p = 0.9743$; Treatment $F(1, 34) = 0.000$, $p > 0.9999$). An effect of side was identified when percentage backward steps were analysed (Interaction $F(1, 34) = 2.193$, $p = 0.1479$; Side $F(1, 34) = 12.84$, $p = 0.0010$; Treatment $F(1, 34) = 0.000$, $p > 0.9999$). Post-hoc comparisons found a difference between ipsilateral and contralateral for the AAV-A53T group ($p < 0.01$) (Figure 4.29).

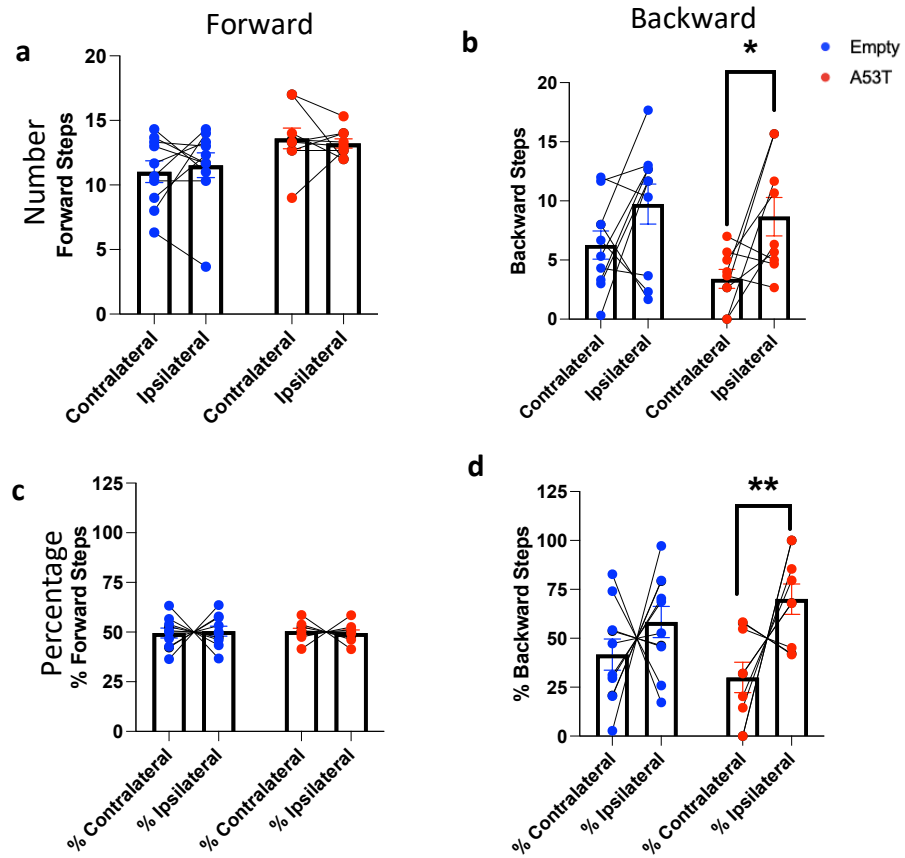


Figure 4.29: Performance in the stepping test 42 days post-AAV delivery for both sexes combined. (b) Decreased contralateral backward steps were identified in the AAV-A53T group when compared to their ipsilateral backward steps. (d) Decreased percentage contralateral backward steps were also identified in the AAV-A53T group when compared to their percentage ipsilateral backward steps. Data expressed mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. $n = 9-10$. * $p < 0.05$, ** $p < 0.01$.

4.5.4.7 No forelimb akinesia was identified in males alone 42 days post-AAV delivery.

No effects were identified between the groups at 42 days post-AAV delivery for males alone in number of forward steps (Interaction $F(1, 14) = 0.2453$, $p = 0.6281$; Side $F(1, 14) = 0.0224$, $p = 0.8831$; Treatment $F(1, 14) = 1.028$, $p = 0.3279$), or number of backward steps (Interaction $F(1, 14) = 1.378$, $p = 0.2600$; Side $F(1, 14) = 3.232$, $p = 0.0938$; Treatment $F(1, 14) = 0.0756$, $p = 0.7874$).

Similarly, no effects were identified between the groups for percentage forward steps (Interaction $F(1, 14) = 2.223$, $p = 0.1582$; Side $F(1, 14) = 0.4509$, $p = 0.5128$; Treatment $F(1, 14) = 0.000$, $p > 0.9999$). Analysis of percentage backward steps identified an interaction effect (Interaction $F(1, 14) = 5.063$, $p = 0.0410$; Side $F(1, 14) = 3.357$, $p = 0.0883$; Treatment $F(1, 14) = 0.000$, $p > 0.9999$). Post-hoc comparisons identified no differences between the treatment groups (Figure 4.30).

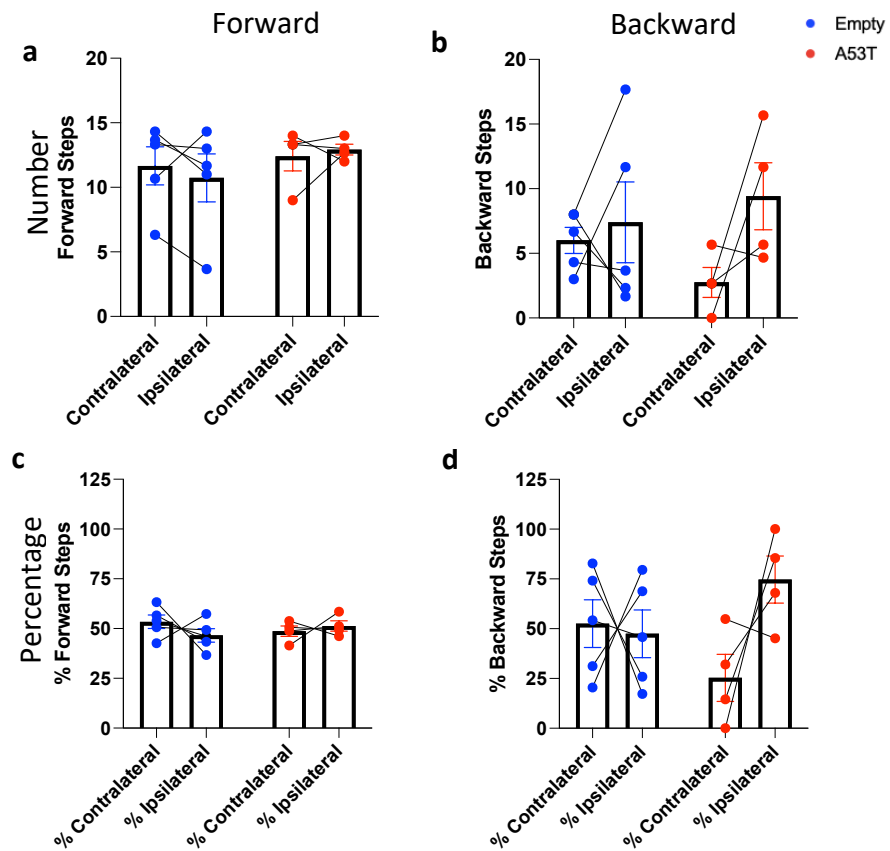


Figure 4.30: Performance in the stepping test 42 days post-AAV delivery for males alone. No differences were identified between the groups in the stepping test at 42 days post-AAV delivery when males were analysed alone. Data expressed mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 4-5.

4.5.4.8 No forelimb akinesia was identified in females alone 42 days post-AAV delivery.

Analysis of the number of forward steps for females alone identified an effect of treatment (Interaction $F(1, 16) = 3.602, p = 0.0759$; Side $F(1, 16) = 0.2719, p = 0.6092$; Treatment $F(1, 16) = 11.00, p = 0.0044$). Post-hoc comparisons found no differences between the treatment groups. An effect of side was identified for number of backward steps (Interaction $F(1, 16) = 0.1611, p = 0.6934$; Side $F(1, 16) = 7.682, p = 0.0136$; Treatment $F(1, 16) = 3.581, p = 0.0767$). Post-hoc comparisons found no differences between the treatment groups.

An interaction effect was identified for percentage forward steps (Interaction $F(1, 16) = 6.471, p = 0.0217$; Side $F(1, 16) = 1.144, p = 0.3007$; Treatment $F(1, 16) = 0.000, p > 0.9999$), with no differences identified between the treatment groups through post-hoc comparisons. Analysis of percentage backward steps identified an effect of side (Interaction $F(1, 16) = 0.0771, p = 0.7849$; Side $F(1, 16) = 11.99, p = 0.0032$; Treatment $F(1, 16) = 0.000, p > 0.9999$). Post-hoc comparisons found no differences between the treatment groups (Figure 4.31).

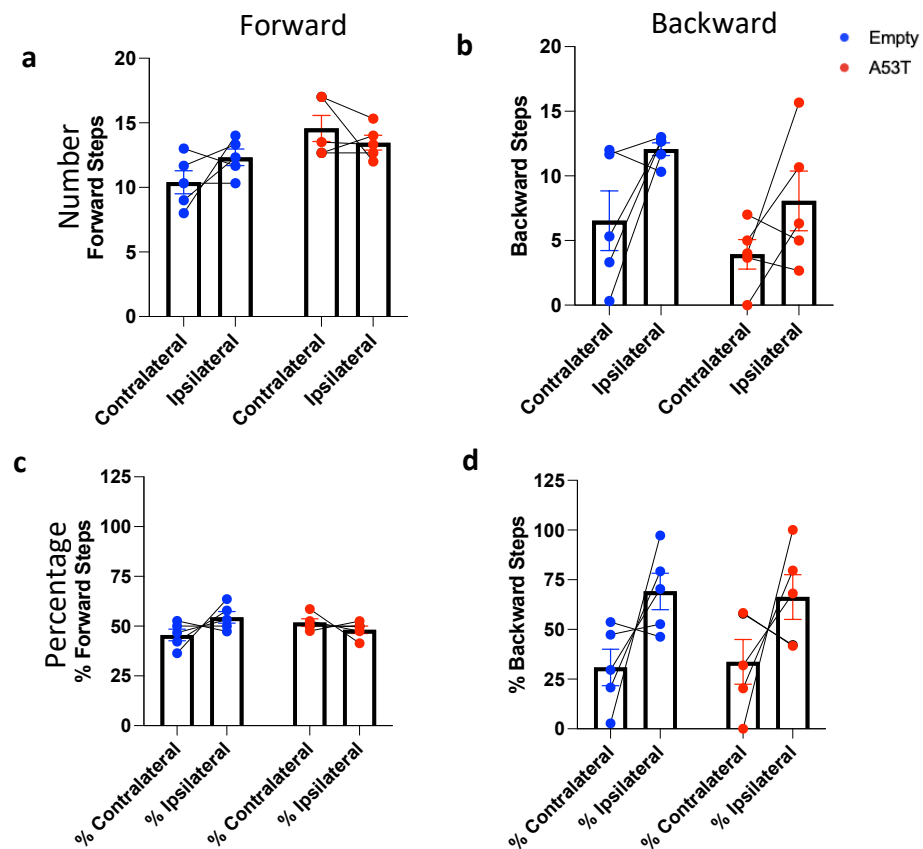


Figure 4.31: Performance in the stepping test 42 days post-AAV delivery for females alone. No differences were identified between the groups in the stepping test at 42 days post-AAV delivery when males were analysed alone. Data expressed mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. $n = 5$.

4.5.5. Tyrosine Hydroxylase Immunoreactive Cells

4.5.5.1 Decreased TH immunoreactive cells in the substantia nigra of the AAV-A53T injected animals were observed when expressed as percentage of the un-injected side and compared to the AAV-empty injected animals, at 6.5 weeks post-AAV delivery.

The number of TH immunoreactive 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. Decreased TH immunoreactive cells were identified in the AAV-A53T group at 6.5 weeks post-AAV delivery when compared with the AAV-empty treatment group ($t(8) = 2.432$, $p = 0.0411$) (Figure 4.32). Due to the lack of persistent behavioural differences observed between the males and females, TH immunoreactive cells were not compared for males and females separately.

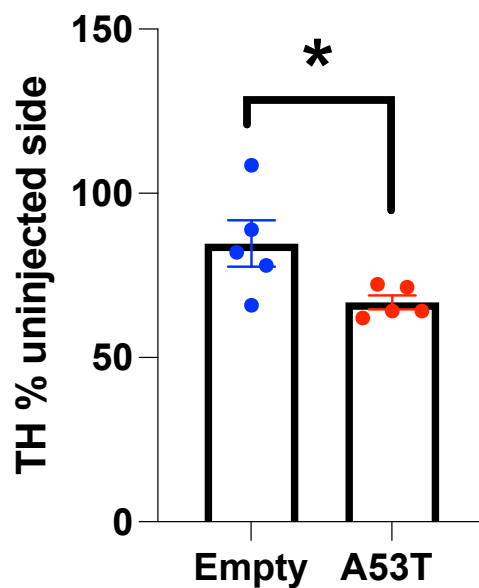


Figure 4.32: Analysis of TH immunoreactive cell counts in the substantia nigra expressed as a percentage of the un-injected side for both sexes combined. Decreased TH immunoreactive cells were identified in the AAV-A53T group when compared to the AAV-empty group at 6.5 weeks post-AAV delivery. Data presented as mean with SEM. Unpaired t test. $n = 5$. * $p < 0.05$.

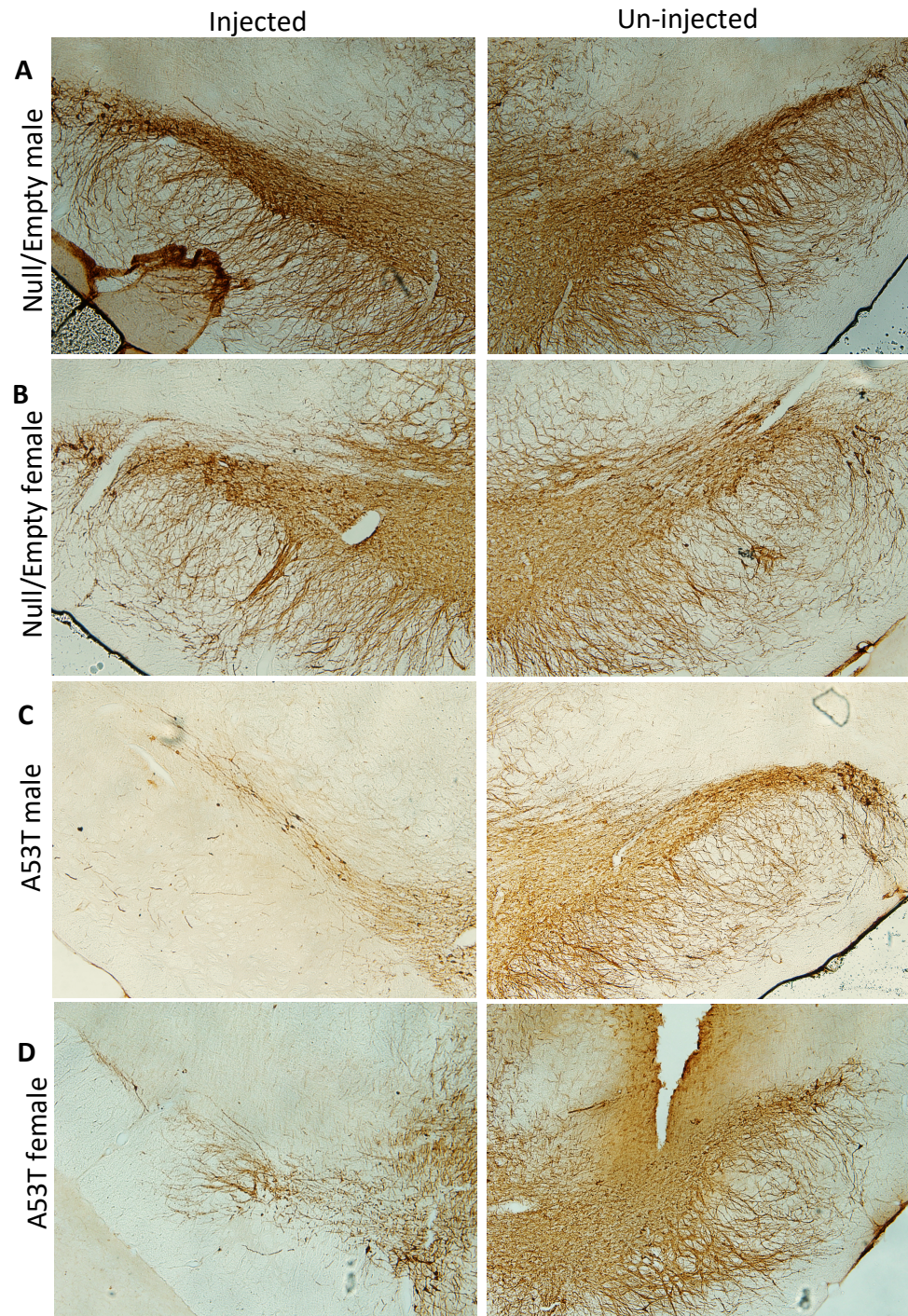


Figure 4.33: Representative images of TH immunoreactivity in the substantia nigra at 6.5 weeks post-AAV delivery in (a) AAV1/2-CMV-Null/Empty injected male, (b) AAV1/2-CMV-Null/Empty injected female, (c) AAV1/2-CMV-A53T alpha-synuclein injected male, (d) AAV1/2-CMV-A53T alpha-synuclein injected female. Images acquired at 10X.

4.5.6 Summary of Results 6.5 Week AAV-A53T Study

	Staircase Test 23 days post- AAV delivery; 43 days post- AAV delivery	Stepping Test 24 days post-AAV delivery; 42 days post-AAV delivery	Cylinder Test 44 days post-AAV delivery	TH immunoreactivity (% un-injected side) 44 days post-AAV delivery
Both Sexes	No differences	24 days: Decreased backward steps in AAV-A53T group. Decreased % backward steps in AAV-A53T and AAV- empty groups. 42 days: Decreased backward steps and % backward steps in AAV-A53T group.	No differences	Decreased in AAV- A53T when compared with AAV-empty.
Male	No differences	24 days: Decreased % backward steps in AAV-A53T and AAV- empty groups. 42 days: No differences	No differences	
Female	No differences	24 days: Decreased backward steps and % backward steps in AAV-A53T group. 42 days: No differences	No differences	

Table 4.6: A summary of the results obtained in the 6.5 week AAV-A53T study.

4.6 Results – 13 week AAV-A53T Study

4.6.1 Alpha-synuclein immunofluorescence

4.6.1.1 Alpha-synuclein immunofluorescence, expressed as arbitrary units, was found to be increased in the alpha-synuclein injected hemisphere when compared with the un-injected hemisphere in the AAV-A53T-alpha-synuclein group at 13 weeks post-AAV delivery.

Comparison of the injected and un-injected hemispheres of the animals in the AAV-A53T group for alpha-synuclein immunofluorescence found a difference between the sides with both sexes combined ($t(19) = 10.44$, $p < 0.0001$), when males were analysed alone ($t(9) = 7.236$, $p < 0.0001$) and when females were analysed alone ($t(9) = 7.163$, $p < 0.0001$) (Figure 4.34).

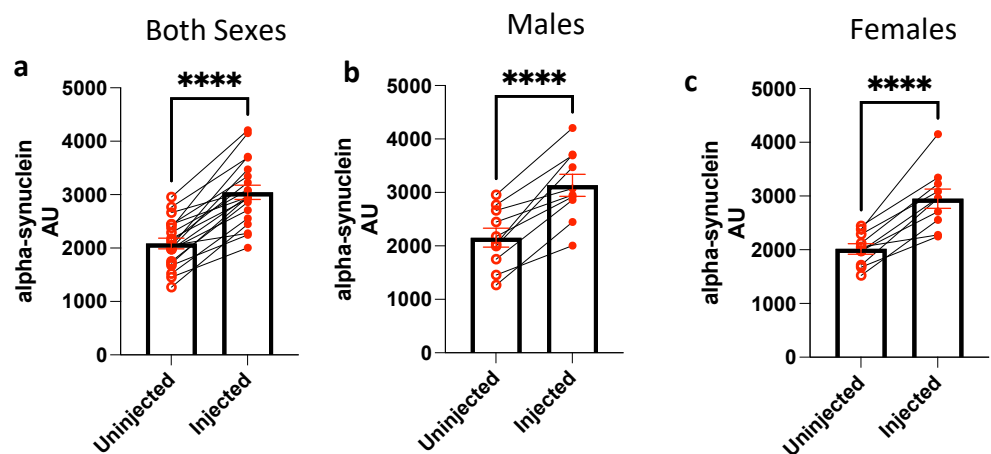


Figure 4.34: Comparison of alpha-synuclein immunofluorescence in the substantia nigra in the injected and un-injected hemispheres of the animals in the AAV-A53T group at 13 weeks post-AAV delivery. Increased alpha-synuclein immunofluorescence was identified in the injected hemisphere when compared with the un-injected hemisphere in the AAV-A53T group. Data presented as mean with SEM. Paired t-test. AU = arbitrary units. $n = 20$ (a) both sexes combined, $n = 10$ (b) males, $n = 10$ (c) females. **** $p < 0.0001$.

4.6.1.2 *Alpha-synuclein immunofluorescence in the substantia nigra, expressed as a percentage of the un-injected side, was found to be increased in the AAV-A53T alpha-synuclein injected animals when compared with the AAV-empty injected animals at 13 weeks post-AAV delivery.*

Analysis of alpha-synuclein immunofluorescence in the substantia nigra as a percentage of the un-injected side in the AAV-empty and AAV-A53T groups found a difference between the groups when both sexes were combined ($t(22.71) = 9.021, p < 0.0001$) and when males were analysed alone ($U=0, p = 0.007$). Due to the low number of samples per group females were not statistically analysed, however their data is presented graphically in Figure 4.35.

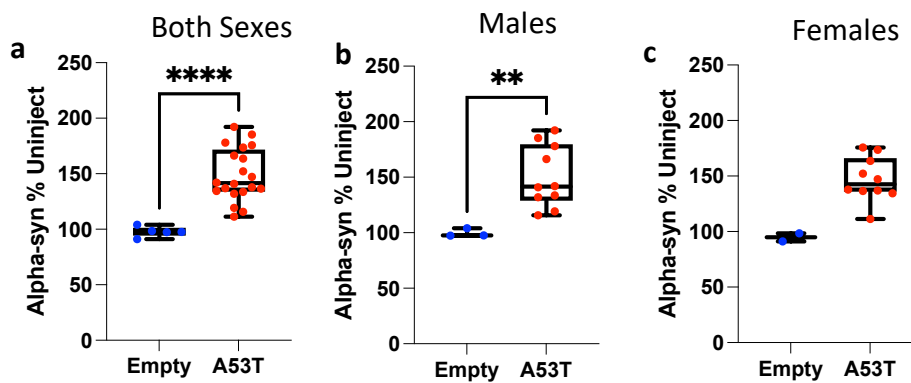


Figure 4.35: Comparison of alpha-synuclein immunofluorescence in the substantia nigra when expressed as a percentage of the un-injected side for the AAV-empty and AAV-A53T groups. Increased alpha-synuclein immunofluorescence was identified in the AAV-A53T group when compared with the AAV-empty group (a, b). Data presented as median with the minimum and maximum. Unpaired t-test with Welch's correction (a) or Mann Whitney U test (b). $n = 5-20$ (a) both sexes combined, $n = 3-10$ (b) males, $n = 2-10$ (c) females. ** $p < 0.01$, **** $p < 0.0001$.

4.6.1.3 Review of the AAV-A53T alpha-synuclein injected animals found alpha-synuclein immunofluorescence in the substantia nigra of all AAV-A53T injected animals at 13 weeks post-AAV delivery.

Alpha-synuclein immunofluorescence was assessed in the substantia nigra of AAV-A53T alpha-synuclein injected animals in both the injected and un-injected hemispheres, as well as in a subset of the AAV-empty injected animals. A number of sections, each 175µm apart, were analysed for expression of alpha-synuclein, as outlined in Table 4.7. Data were assessed as the alpha-synuclein immunofluorescence in the un-injected side divided by the alpha-synuclein immunofluorescence in the injected side.

Animal	Sex	Treatment Group	Expression over 100% (number of sections)
15	Female	A53T	13/13
16	Male	A53T	7/7
17	Male	A53T	12/12
18	Male	A53T	11/11
19	Male	A53T	6/6
20	Male	A53T	14/14
7	Male	A53T	3/3
1	Female	A53T	4/4
14	Female	A53T	6/6
2	Female	A53T	5/5
3	Female	A53T	5/5
4	Female	A53T	8/8
5	Female	A53T	11/11
6	Male	A53T	3/3
8	Male	A53T	2/2
9	Male	A53T	4/4
10	Male	A53T	7/7
11	Female	A53T	5/5
12	Female	A53T	7/7
13	Female	A53T	7/7
25	Male	Empty	2/5
26	Male	Empty	4/9
29	Male	Empty	3/6
31	Female	Empty	4/5
30	Female	Empty	3/10

Table 4.7: Analysis of alpha-synuclein immunofluorescence in the injected and un-injected hemisphere of the substantia nigra of animals in the AAV-A53T alpha-synuclein and AAV-empty groups. Expression over 100% represents alpha-synuclein immunofluorescence in the injected hemisphere expressed as percentage of the un-injected side, that is greater than 100%. The data represented in this table are taken from a number of sections from the substantia nigra.

Representative images of alpha-synuclein and TH positive immunofluorescence in the substantia nigra of male and female AAV1/2-CMV-A53T alpha-synuclein (human) treated animals (Figure 4.36).

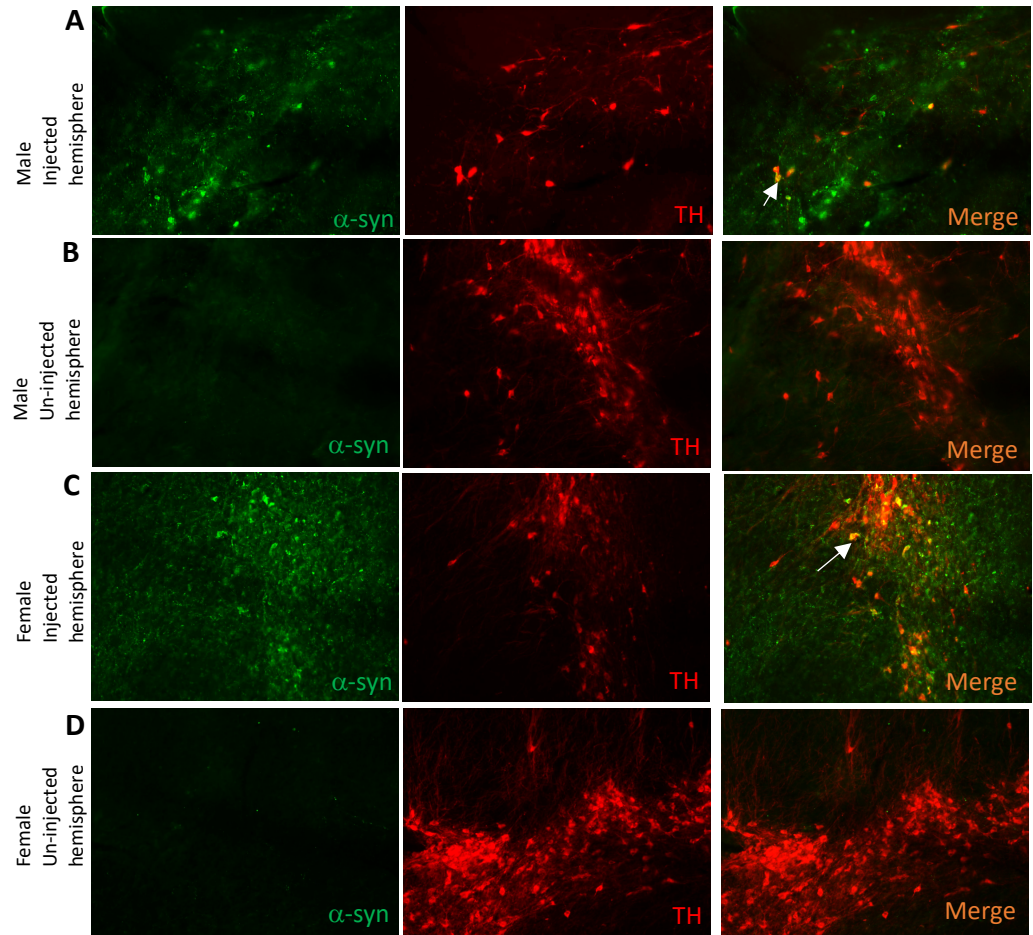


Figure 4.36: Representative images of alpha-synuclein and TH immunofluorescence in the substantia nigra 13 weeks post-AAV delivery. (A) A53T alpha-synuclein injected male injected hemisphere, (B) A53T alpha-synuclein injected male un-injected hemisphere, (C) A53T alpha-synuclein injected female injected hemisphere, (D) A53T alpha-synuclein injected female un-injected hemisphere. First column represents alpha-synuclein immunofluorescence, second column represents TH immunofluorescence, third column represents merged images of the first 2 columns. Images acquired at 20X. Arrows indicate co-expression of alpha-synuclein and TH.

4.6.1.4 All animals injected with AAV-A53T-alpha-synuclein expressed higher levels of alpha-synuclein immunofluorescence than AAV-empty injected animals.

Reviewing the immunofluorescence of alpha-synuclein in the AAV-A53T group as a percentage of the un-injected side found no animals expressing alpha-synuclein immunofluorescence levels in the injected hemisphere less than 100% of the un-injected side. Due to the inclusion of the AAV-empty group in the alpha-synuclein immunofluorescence analysis, the mean, standard deviations and SEM was calculated when the data were presented as percentage of the un-injected hemisphere for both the AAV-A53T and AAV-empty groups.

Group	Number of Values	Minimum	Maximum	Range	Mean	Standard Deviation	SEM
Empty	5	91.23	104.1	12.91	97.73	4.581	2.049
A53T	20	111.3	192.3	81.02	148.8	23.58	5.272

Table 4.8: Descriptive statistics for alpha-synuclein immunofluorescence in the substantia nigra as a percentage of the un-injected side in the AAV-empty and AAV-A53T groups.

Reviewing the data, there were no animals in the AAV-A53T group identified whose alpha-synuclein immunofluorescence as percentage of the un-injected side fell within the mean $\pm$ the standard deviation of the AAV-empty group and therefore all animals were included in analysis.

4.6.2 Staircase Test

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

Two animals from the Empty group and five animals from the A53T group met the criteria for unsuccessful acquisition of the Staircase test as outlined in Table 2.2 and were removed from all analysis. No differences were identified at baseline between the groups when both sexes were analysed together and when both sexes were analysed separately. Data presented in Appendix II.

4.6.2.2 No differences were identified between the groups in the staircase test at 30-33 days post-AAV delivery.

No effects were identified when both sexes were combined (Interaction $F(1, 60) = 3.519, p = 0.0655$; Side $F(1, 60) = 1.773, p = 0.1880$; Treatment $F(1, 60) = 0.3761, p = 0.5420$), or when females were analysed alone (Interaction $F(1, 28) = 0.000, p > 0.9999$; Side $F(1, 60) = 0.4684, p = 0.4993$; Treatment $F(1, 60) = 0.4684, p = 0.4993$) in the staircase test at 30-33 days post-AAV delivery. An interaction effect was identified when males were analysed alone (Interaction $F(1, 28) = 4.985, p = 0.0337$; Side $F(1, 28) = 1.643, p = 0.2104$; Treatment $F(1, 28) = 0.0987, p = 0.7558$). Post-hoc comparisons identified no differences between the treatment groups (Figure 4.37).

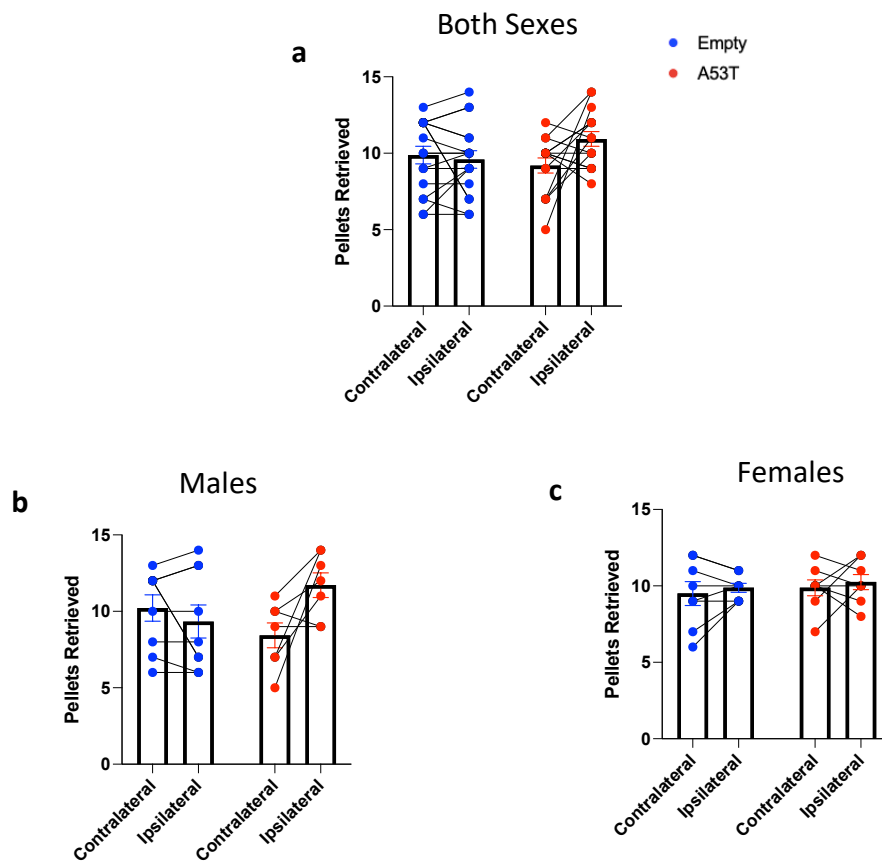


Figure 4.37: Number of pellets retrieved at 30-33 days post-AAV delivery in the staircase test. No differences were identified between the treatment groups at 30-33 days post-AAV delivery in the staircase test. Data presented as mean with SEM. Two-Way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 15 – 17 (a) both sexes combined, n = 7 – 9 (b) males, n = 8 (c) females.

4.6.2.3 No differences were identified between the groups in the staircase test at 48-51 days post-AAV delivery.

When analysed at 48-51 days post-AAV delivery no effects were identified with both sexes combined for number of pellets retrieved (Interaction $F(1, 60) = 0.8510$, $p = 0.3600$; Side $F(1, 60) = 0.0124$, $p = 0.9116$; Treatment $F(1, 60) = 1.873$, $p = 0.1762$), for males alone (Interaction $F(1, 28) = 0.7824$, $p = 0.3839$; Side $F(1, 28) = 0.5729$, $p = 0.4554$; Treatment $F(1, 28) = 2.432$, $p = 0.1301$), or for females alone (Interaction $F(1, 28) = 0.2184$, $p = 0.6439$; Side $F(1, 28) = 0.3882$, $p = 0.5383$; Treatment $F(1, 28) = 0.0971$, $p = 0.7577$) (Figure 4.38).

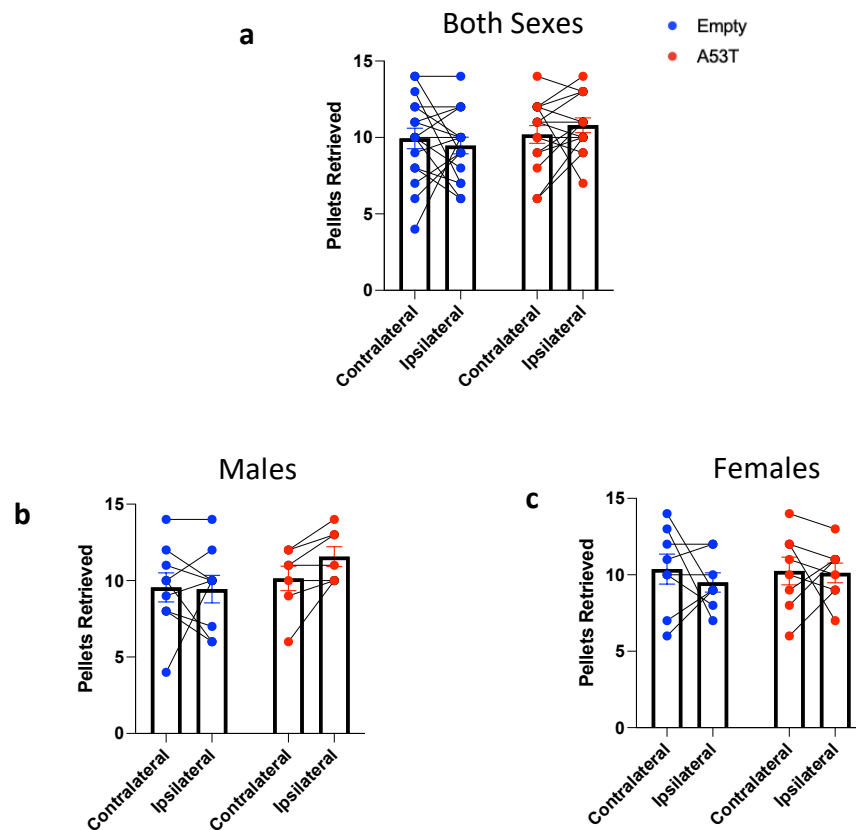


Figure 4.38: Number of pellets retrieved at 48-51 days post-AAV delivery in the staircase test. No differences were identified between the treatment groups at 48-51 days post-AAV delivery in the staircase test. Data presented as mean with SEM. Two-Way ANOVA. $n = 15 - 17$ (a) both sexes combined, $n = 7 - 9$ (b) males, $n = 8$ (c) females.

4.6.2.4 No differences were identified between the groups in the staircase test at 61-64 days post-AAV delivery.

At 61-64 days post-AAV delivery the staircase test was repeated, with each animal exposed to two trials over two days. On the first day the ipsilateral side of the staircase was baited only, with the contralateral side left empty. On the second day the contralateral side of the staircase was baited only, leaving the ipsilateral side empty. This is a modified version of the staircase test known as the side test (Montoya et al., 1991). This version of the test is employed in order to evaluate if any side differences observed are due to the animal directing its attention or effort primarily to one side as opposed to greater motor skill on one side over the other. No differences were found for number of ipsilateral pellets retrieved for both sexes combined ($U = 114$, $p = 0.6133$), males alone ($U = 29$, $p = 0.8051$), or females alone ($t(14) = 0.7361$, $p = 0.4738$). Similarly no differences were found for number of contralateral pellets retrieved for both sexes combined ($t(30) = 0.2913$, $p = 0.7728$), for males alone ($t(14) = 0.1005$, $p = 0.9213$), or females for alone ($t(14) = 0.4157$, $p = 0.6839$) (Figure 4.39).

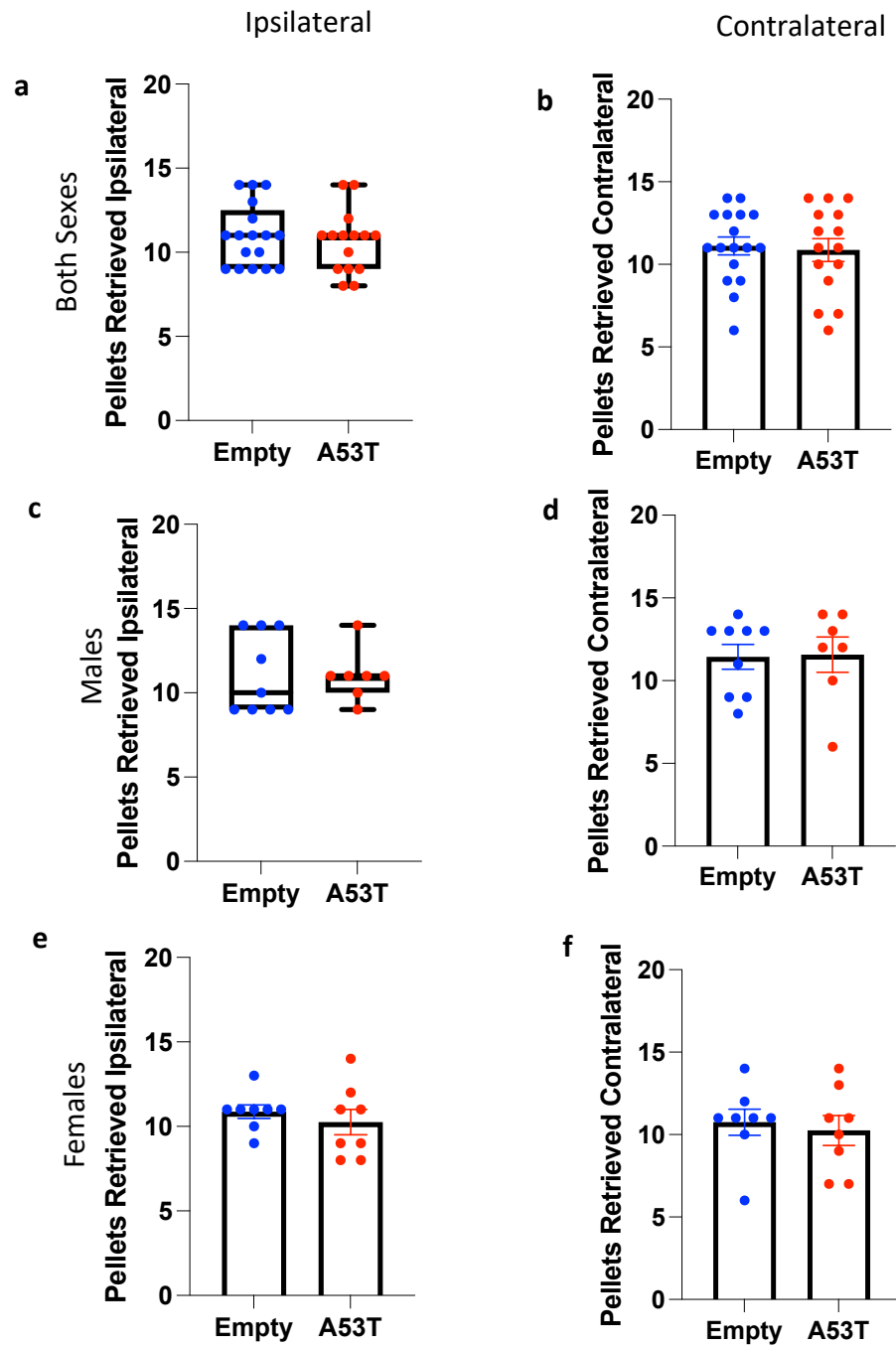


Figure 4.39: Number of pellets retrieved on the ipsilateral and contralateral sides at 61-64 days post-AAV delivery in the staircase test. No differences were identified between the treatment groups at 61-64 days post-AAV delivery in the staircase test. Data presented as median with minimum and maximum (a, c) or mean with SEM (b, d, e, f). Mann Whitney test or unpaired t-test. $n = 15 - 17$ (a, b) both sexes combined, $n = 7 - 9$ (c, d) males, $n = 8$ (e, f) females.

4.6.2.5 No differences were identified between the groups in the staircase test at 76-78 days post-AAV delivery.

When analysed at 76-78 days post-AAV delivery no effects were identified with both sexes combined for number of pellets retrieved (Interaction $F(1, 60) = 0.014$, $p = 0.9063$; Side $F(1, 60) = 1.644$, $p = 0.2047$; Treatment $F(1, 60) = 1.644$, $p = 0.2047$) or for females alone (Interaction $F(1, 28) = 0.2042$, $p = 0.6548$; Side $F(1, 28) = 1.452$, $p = 0.2383$, Treatment $F(1, 28) = 0.8169$, $p = 0.3738$). When males were analysed alone an effect of side was identified (Interaction $F(1, 28) = 0.1237$, $p = 0.7277$; Side $F(1, 28) = 9.103$, $p = 0.0054$; Treatment $F(1, 28) = 0.1511$, $p = 0.7004$). Post-hoc comparisons did not find any differences between the treatment groups.

Analysis of the percentage success at 76-78 days post-AAV delivery found no effects for both sexes combined (Interaction $F(1, 58) = 1.394$, $p = 0.2426$; Side $F(1, 58) = 2.112$, $p = 0.1516$; Treatment $F(1, 58) = 0.1468$, $p = 0.7030$), for males alone (Interaction $F(1, 26) = 1.929$, $p = 0.1766$; Side $F(1, 26) = 2.643$, $p = 0.1161$; Treatment $F(1, 26) = 0.1422$, $p = 0.7091$) or for females alone (Interaction $F(1, 28) = 0.0238$, $p = 0.8785$; Side $F(1, 28) = 0.0949$, $p = 0.7604$; Treatment $F(1, 28) = 0.7216$, $p = 0.4028$) (Figure 4.40).

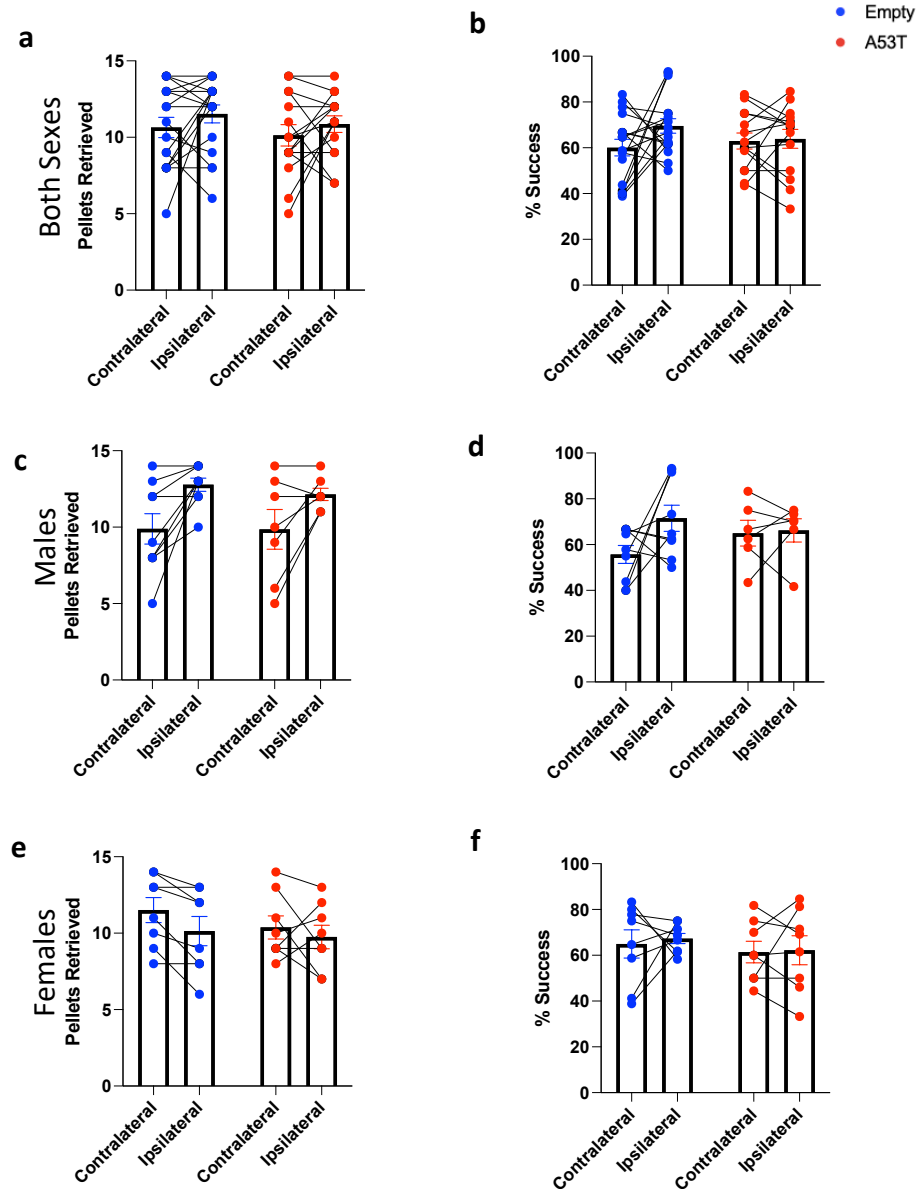


Figure 4.40: Number of pellets retrieved and percentage success at 76-78 days post-AAV delivery in the staircase test. No differences were identified between the treatment groups at 76-78 days post-AAV delivery in the staircase test. Data presented as mean with SEM. Two-Way ANOVA followed by Neuman-Keuls multiple comparisons test. $n = 15 - 17$ (a, b) both sexes combined, $n = 7 - 9$ (c, d) males, $n = 8$ (e, f) females.

4.6.2.6 No differences were identified between the groups in the staircase test at 83 days post-AAV delivery.

At 83 days post-AAV delivery the animals were exposed to the staircase test for one final testing session. During this 10 minute trial, only the contralateral side was baited. No differences were identified between the groups for number of pellets retrieved when both sexes were combined ($U=115.5$, $p = 0.6551$), for males alone ($U = 29.5$, $p = 0.8832$) or for females alone ($t(14) = 0.3073$, $p = 0.7631$). Similarly, no differences were identified for percentage success when both sexes were analysed together ($t(30) = 1.129$, $p = 0.2677$), for males alone ($U = 15.50$, $p = 0.095$), or for females alone ($t(14) = 0.078$, $p = 0.9392$) (Figure 4.41).

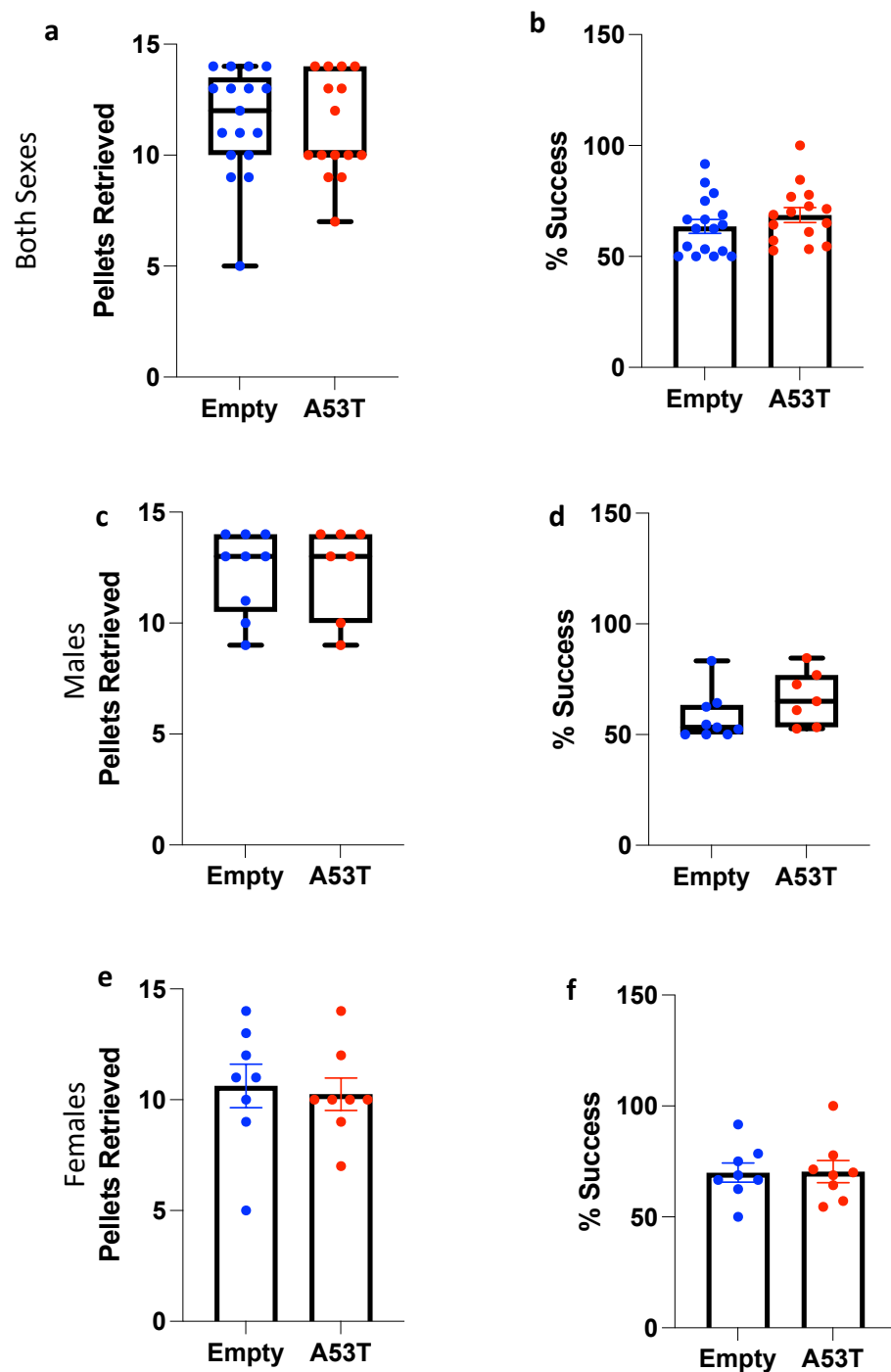


Figure 4.41: Number of pellets retrieved and percentage success at 83 days post-AAV delivery in the staircase test. No differences were identified between the treatment groups at 76-78 days post-AAV delivery in the staircase test. Data presented as mean with SEM (b, e, f) and median with minimum and maximum (a, c, d). Unpaired t-test (b, e, f) or Mann Whitney test (a, c, d). $n = 15 - 17$ (a, b) both sexes combined, $n = 7 - 9$ (c, d) males, $n = 8$ (e, f) females.

4.6.3 Cylinder Test

4.6.3.1 Decreased percentage contralateral wall contacts were observed in the AAV-A53T group at 50-52 days post-AAV delivery when both sexes were combined.

A difference was identified between the groups for percentage contralateral wall contacts ($t(37) = 2.055$, $p = 0.0470$) at 50-52 days post-AAV delivery. No differences were identified at 50-52 days post-AAV delivery for number of contralateral wall contacts ($t(37)=1.295$, $p = 0.2034$), number of ipsilateral wall contacts ($U = 125.5$, $p = 0.0705$), percentage ipsilateral wall contacts ($t(37) = 1.437$, $p = 0.1592$), number of simultaneous wall contacts ($t(37) = 0.5200$, $p = 0.6062$), or percentage simultaneous wall contacts ($t(37) = 0.3405$, $p = 0.7354$) (Figure 4.42).

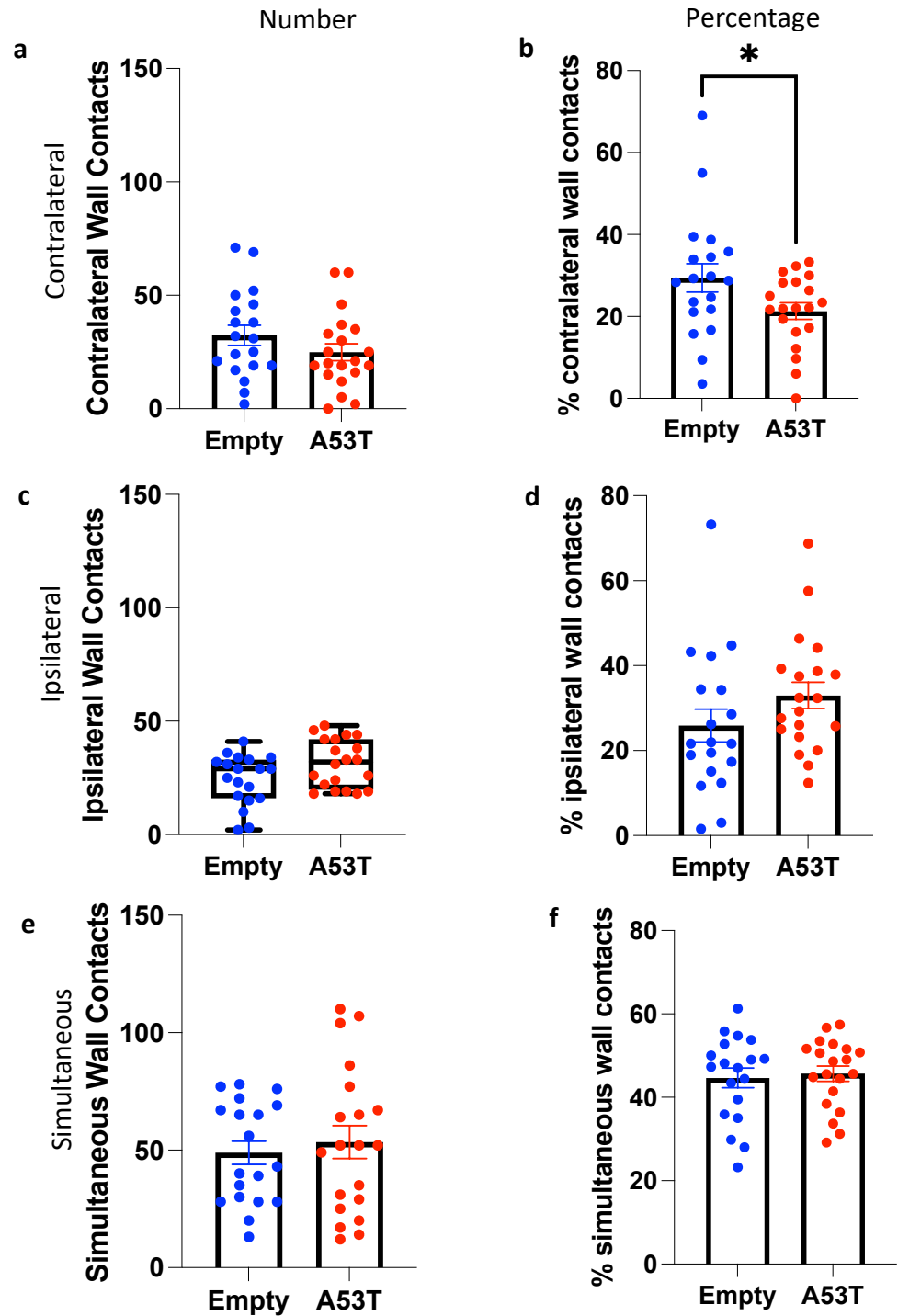


Figure 4.42: Performance in the cylinder test at 50-52 days post-AAV delivery for both sexes combined. Decreased percentage contralateral wall contacts in the AAV-A53T group were identified when compared with the percentage contralateral wall contacts in the AAV-empty group (b). Data presented as mean 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 = 19-20. * $p < 0.05$.

4.6.3.2 Decreased contralateral wall contacts and increased percentage ipsilateral wall contacts were observed in the AAV-A53T group at 50-52 days post-AAV delivery when males were analysed alone.

When males were analysed alone at 50-52 days post-AAV delivery a difference between the groups was identified for number of contralateral wall contacts ($t(18) = 2.510$, $p = 0.0219$), percentage contralateral wall contacts ($t(18) = 2.226$, $p = 0.0390$), and percentage ipsilateral wall contacts ($t(18) = 2.164$, $p = 0.0441$). No differences were identified between the groups for number of ipsilateral wall contacts ($U = 25$, $p = 0.0605$), number of simultaneous wall contacts ($U = 34.50$, $p = 0.2541$), or percentage simultaneous wall contacts ($t(18) = 0.4725$, $p = 0.6423$) (Figure 4.43).

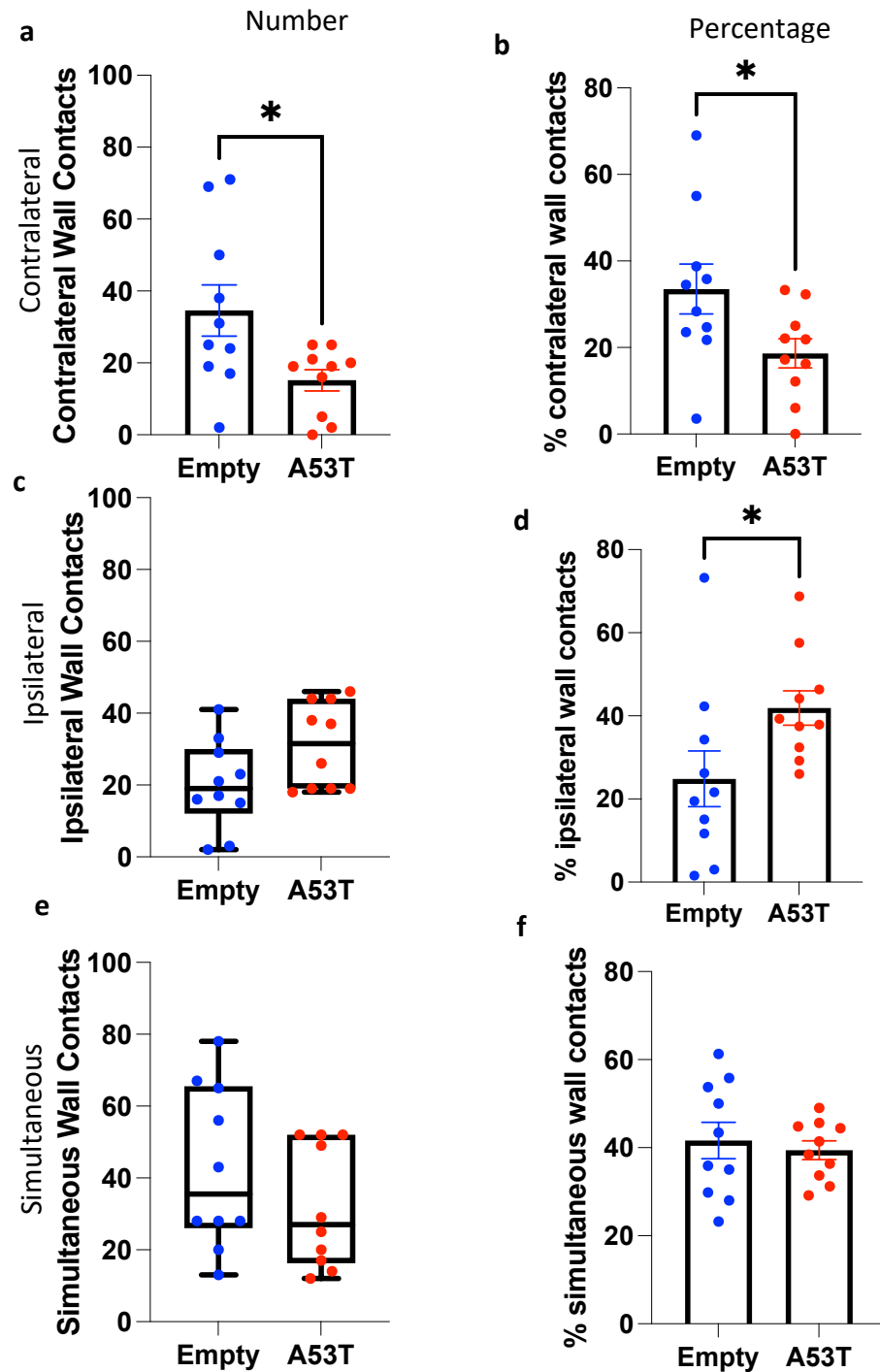


Figure 4.43: Performance in the cylinder test at 50-52 days post-AAV delivery for males alone. Decreased number and percentage of contralateral wall contacts in the AAV-A53T males were identified when compared with the number and percentage of contralateral wall contacts in the AAV-empty males. Increased percentage ipsilateral wall contacts were identified in the AAV-A53T males when compared with the AAV-empty males (a, b). Data presented as mean with SEM (a, b, d, f) or median with minimum and maximum (c, e). Unpaired t-test or Mann Whitney test. $n = 10$. * $p < 0.05$.

4.6.3.3 Increased percentage simultaneous wall contacts were observed in the AAV-A53T group at 50-52 days post-AAV delivery when females were analysed alone.

When females were analysed alone at 50-52 days post-AAV delivery a difference between the groups was identified for percentage simultaneous wall contacts ($t(17) = 2.131, p = 0.0480$). No differences were identified between the groups for number of contralateral wall contacts ($t(17) = 0.6205, p = 0.5432$), percentage contralateral wall contacts ($t(17) = 0.2461, p = 0.8085$), number of ipsilateral wall contacts ($U = 41.50, p = 0.7953$), percentage ipsilateral wall contacts ($t(17) = 0.6675, p = 0.5134$) or number of simultaneous wall contacts ($t(17) = 1.675, p = 0.1121$) (Figure 4.44).

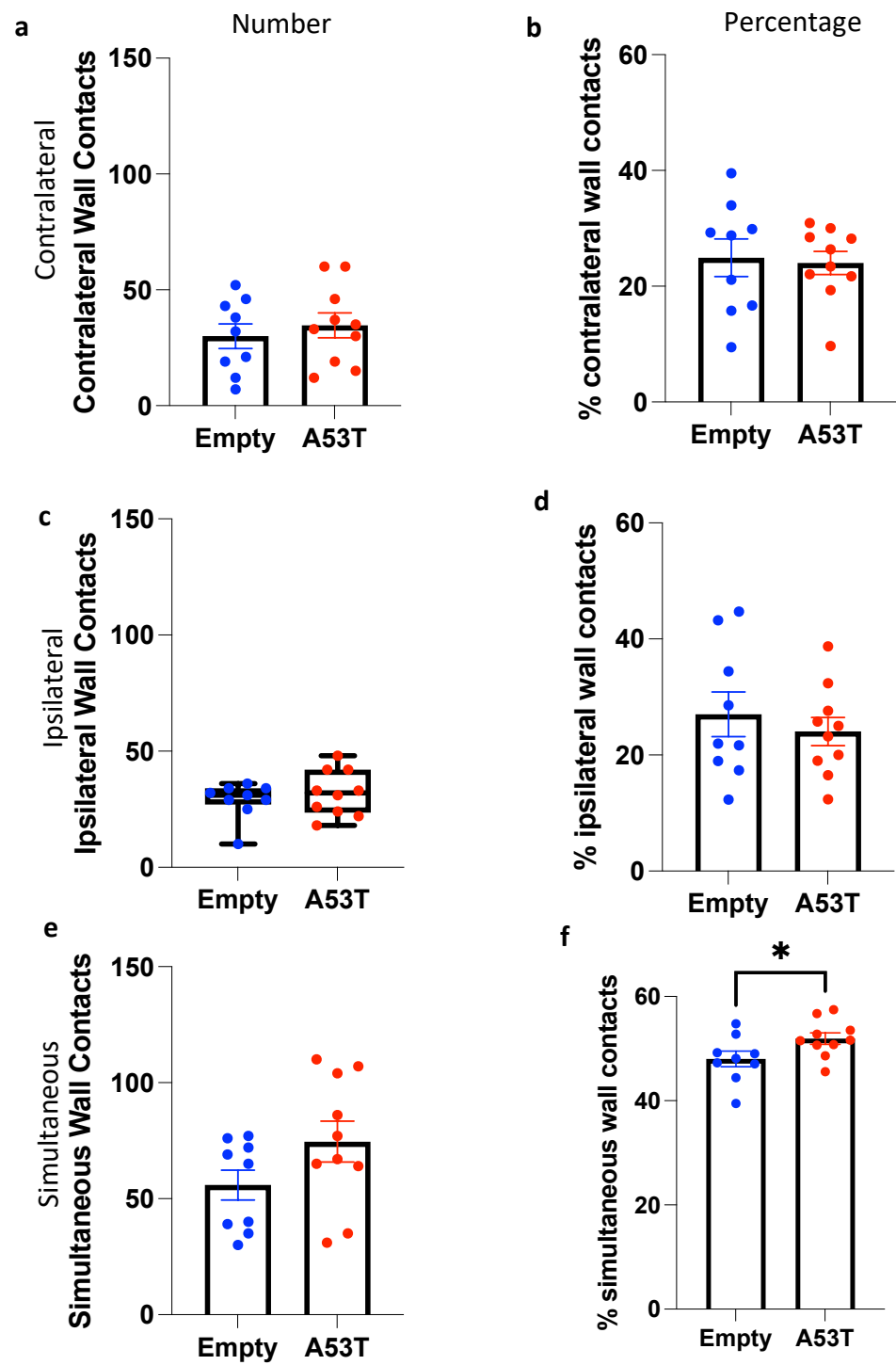


Figure 4.44: Performance in the cylinder test at 50-52 days post-AAV delivery for females alone. Increased percentage simultaneous wall contacts were identified in the AAV-A53T females when compared with the AAV-empty females (f). Data presented as mean with SEM (a, b, d, e, f) or median with minimum and maximum (c). Unpaired t-test (a, b, d, e, f) or Mann Whitney test (c). n = 9-10. * $p < 0.05$.

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

Analysis of the floor landings at 50-52 days post-AAV delivery identified no differences between the groups. Data presented in Appendix II.

4.6.3.5 Limb use asymmetry was found when both sexes were analysed together and when males were analysed alone at 50-52 days post-AAV delivery.

A difference was identified between the groups in limb use asymmetry at 50-52 days post-AAV delivery with both sexes combined ($t(37) = 2.208$, $p = 0.0335$) and when males were analysed alone ($t(18) = 3.034$, $p = 0.0071$). No differences were identified when females were analysed alone ($t(17) = 0.2242$, $p = 0.8252$) (Figure 4.45).

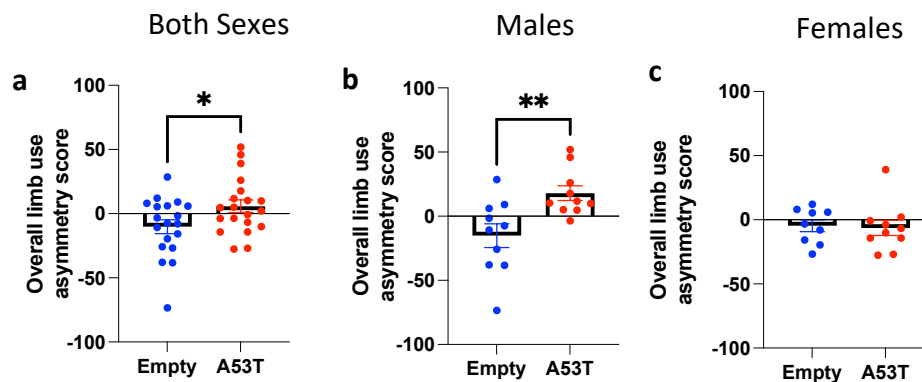


Figure 4.45: Limb use asymmetry 50-52 days post-AAV delivery in the cylinder test. Increased limb use asymmetry was observed in the AAV-A53T group when both sexes were combined, and when males were analysed alone at 50-52 days post-AAV delivery. Data presented as mean with SEM. Unpaired t-test. $n = 19 - 20$ (a) both sexes combined, $n = 10$ (b) males, $n = 9-10$ (c) females. * $p < 0.05$, ** $p < 0.01$

4.6.3.6 Limb use asymmetry for wall alone was found when males were analysed alone at 50-52 days post-AAV delivery.

Limb use asymmetry was also analysed for wall and floor separately.

A difference was identified between the groups in limb use asymmetry for wall at 50-52 days post-AAV delivery for males alone ($t(18) = 2.306$, $p = 0.0333$), with no differences identified with both sexes combined ($t(37) = 1.803$, $p = 0.0795$) or when females were analysed alone ($t(17) = 0.2565$, $p = 0.8007$) (Figure 4.46).

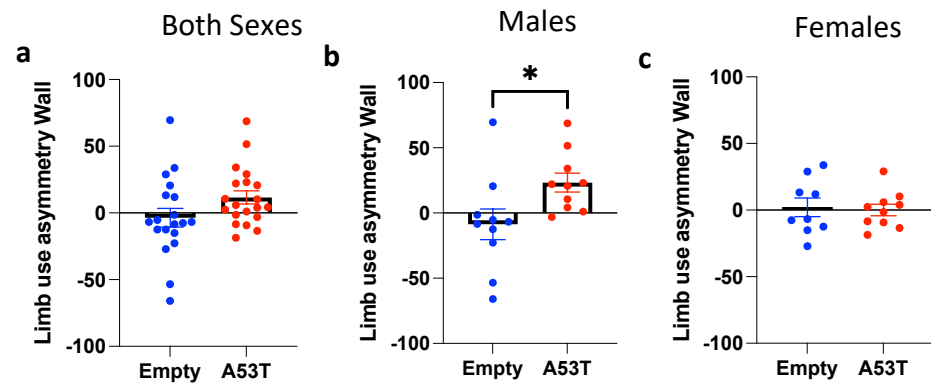


Figure 4.46: Limb use asymmetry for wall at 50-52 days post-AAV delivery in the cylinder test. Increased limb use asymmetry for wall was observed in the AAV-A53T males when compared with the AAV-empty males at 50-52 days post-AAV delivery (b). Data presented as mean with SEM. Unpaired t-test. $n = 19 - 20$ (a) both sexes combined, $n = 10$ (b) males, $n = 9-10$ (c) females. * $p < 0.05$.

4.6.3.7 Limb use asymmetry was not observed when floor was analysed alone at 50-52 days post-AAV delivery.

No differences were identified between the groups in limb use asymmetry for floor at 50-52 days post-AAV delivery for both sexes combined ($t(37) = 1.241$, $p = 0.2224$), for males alone ($t(18) = 1.540$, $p = 0.1409$), or for females alone ($t(17) = 0.0934$, $p = 0.9267$) (Figure 4.47).

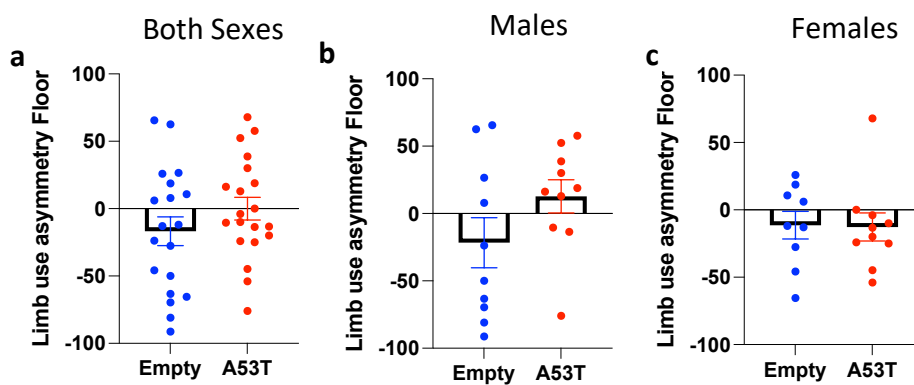


Figure 4.47: Limb use asymmetry for floor at 50-52 days post-AAV delivery in the cylinder test. Limb use asymmetry for floor was not found at 50-52 days post-AAV delivery in the cylinder test. Data presented as mean with SEM. Unpaired t-test. $n = 19 - 20$ (a) both sexes, $n = 10$ (b) males, $n = 9-10$ (c) females.

4.6.3.8 No differences were identified at 86 days post-AAV delivery in the cylinder test with both sexes combined.

The cylinder test was repeated at 86 days post-AAV delivery. No differences were identified with both sexes combined for number of contralateral wall contacts ($U = 171, p = 0.6014$), percentage contralateral wall contacts ($U = 175, p = 0.6818$), number of ipsilateral wall contacts ($U = 173.5, p = 0.6512$), percentage ipsilateral wall contacts ($t(37) = 0.5517, p = 0.5845$), number of simultaneous wall contacts ($U = 181.5, p = 0.8187$), or percentage simultaneous wall contacts ($t(37) = 0.2676, p = 0.7905$) (Figure 4.48).

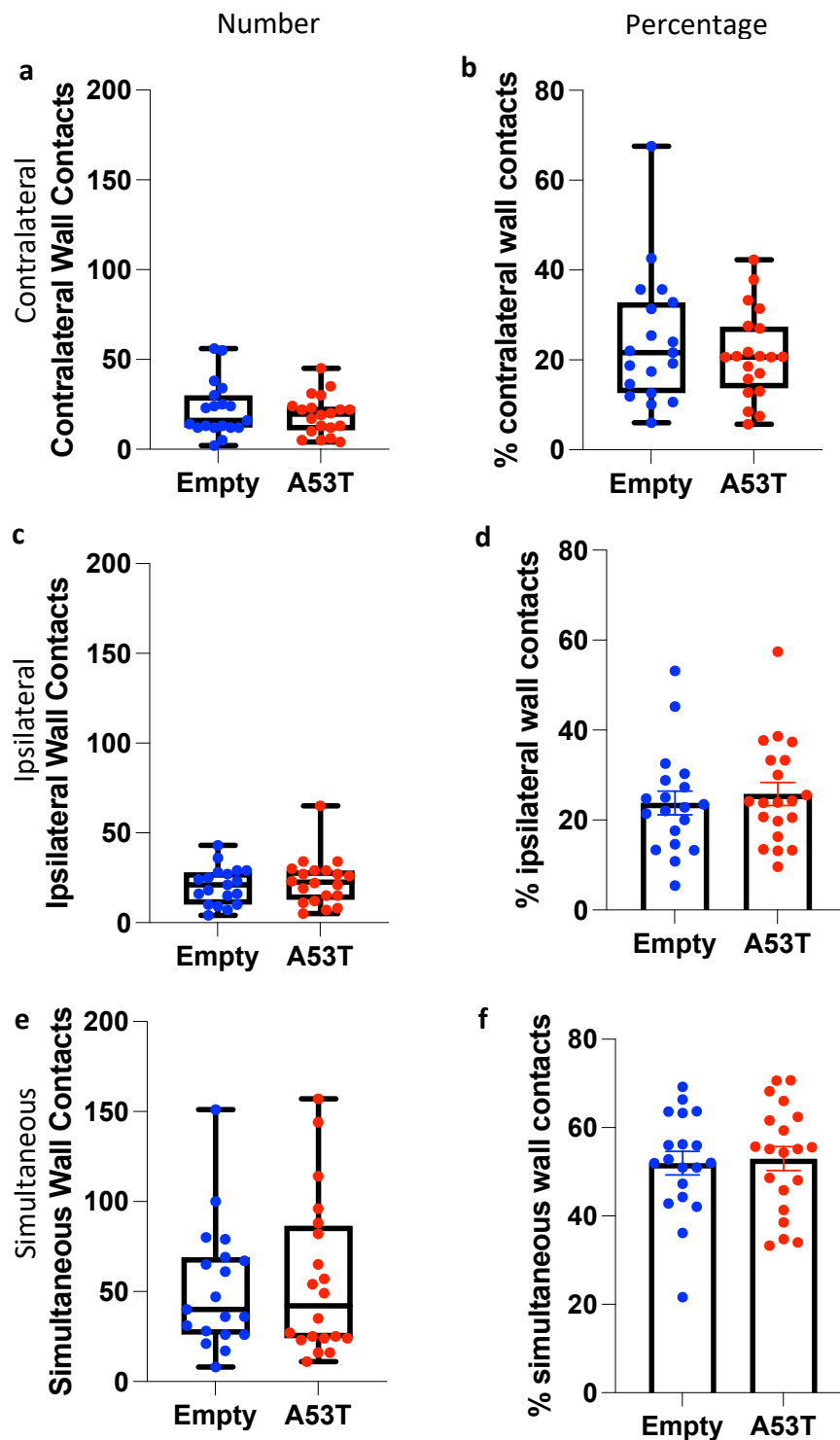


Figure 4.48: Performance in the cylinder test at 86 days post-AAV delivery for both sexes combined. No differences were observed between the treatment groups at 86 days post-AAV delivery in the cylinder test when males and females were analysed together. Data presented as mean with SEM (d, f) or median with minimum and maximum (a, b, c, e). Unpaired t-test (d, f) or Mann Whitney test (a, b, c, e). n = 19-20.

4.6.3.9 No differences were identified at 86 days post-AAV delivery in the cylinder test when males were analysed alone.

No differences at 86 days post-AAV delivery were identified when males were analysed alone for number of contralateral limb contacts ($U = 43.50$, $p = 0.6420$), percentage contralateral limb contacts ($t(18) = 0.3524$, $p = 0.7286$), number of ipsilateral limb contacts ($t(18) = 0.8173$, $p = 0.4244$), percentage ipsilateral limb contacts ($t(18) = 1.098$, $p = 0.2865$), number of simultaneous limb contacts ($U = 32$, $p = 0.1832$), or percentage simultaneous limb contacts ($t(18) = 0.8438$, $p = 0.4099$) (Figure 4.49).

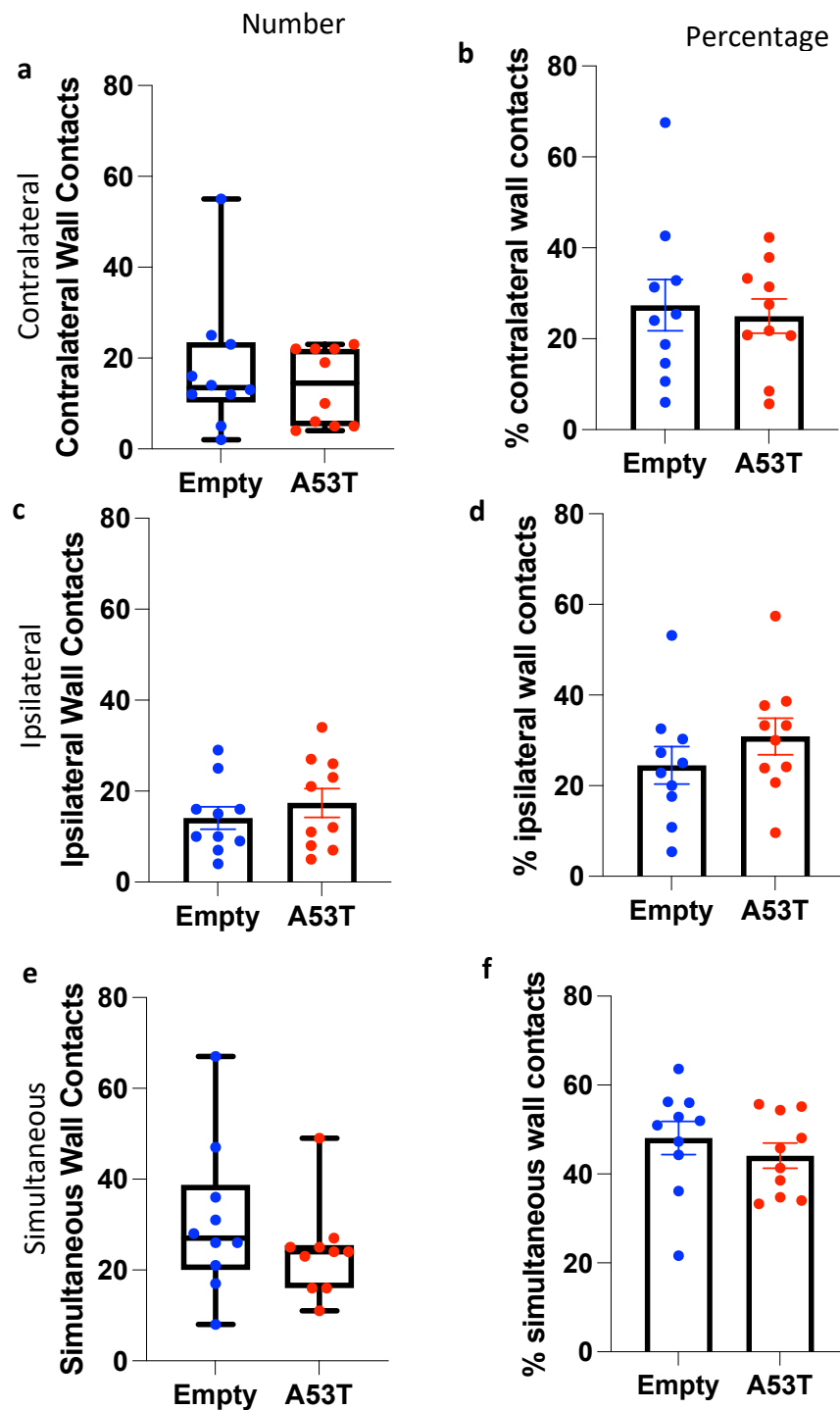


Figure 4.49: Performance in the cylinder test at 86 days post-AAV delivery for males alone. No differences were observed between the treatment groups at 86 days post-AAV delivery in the cylinder test when males were analysed alone. Data presented as mean with SEM (b, c, d, f) or median with minimum and maximum (a, e). Unpaired t-test (b, c, d, f) or Mann Whitney test (a, e). n = 10.

4.6.3.10 No differences were identified at 86 days post-AAV delivery in the cylinder test when females were analysed alone.

No differences at 86 days post-AAV delivery were identified when females were analysed alone for number of contralateral wall contacts ($t(17) = 0.5104$, $p = 0.6163$), percentage contralateral wall contacts ($t(17) = 0.9491$, $p = 0.3559$), number of ipsilateral wall contacts ($U = 43.50$, $p = 0.9202$), percentage ipsilateral wall contacts ($t(17) = 0.5588$, $p = 0.5836$), number of simultaneous wall contacts ($t(17) = 0.7900$, $p = 0.4404$), or percentage simultaneous wall contacts ($t(17) = 1.395$, $p = 0.1809$) (Figure 4.50).

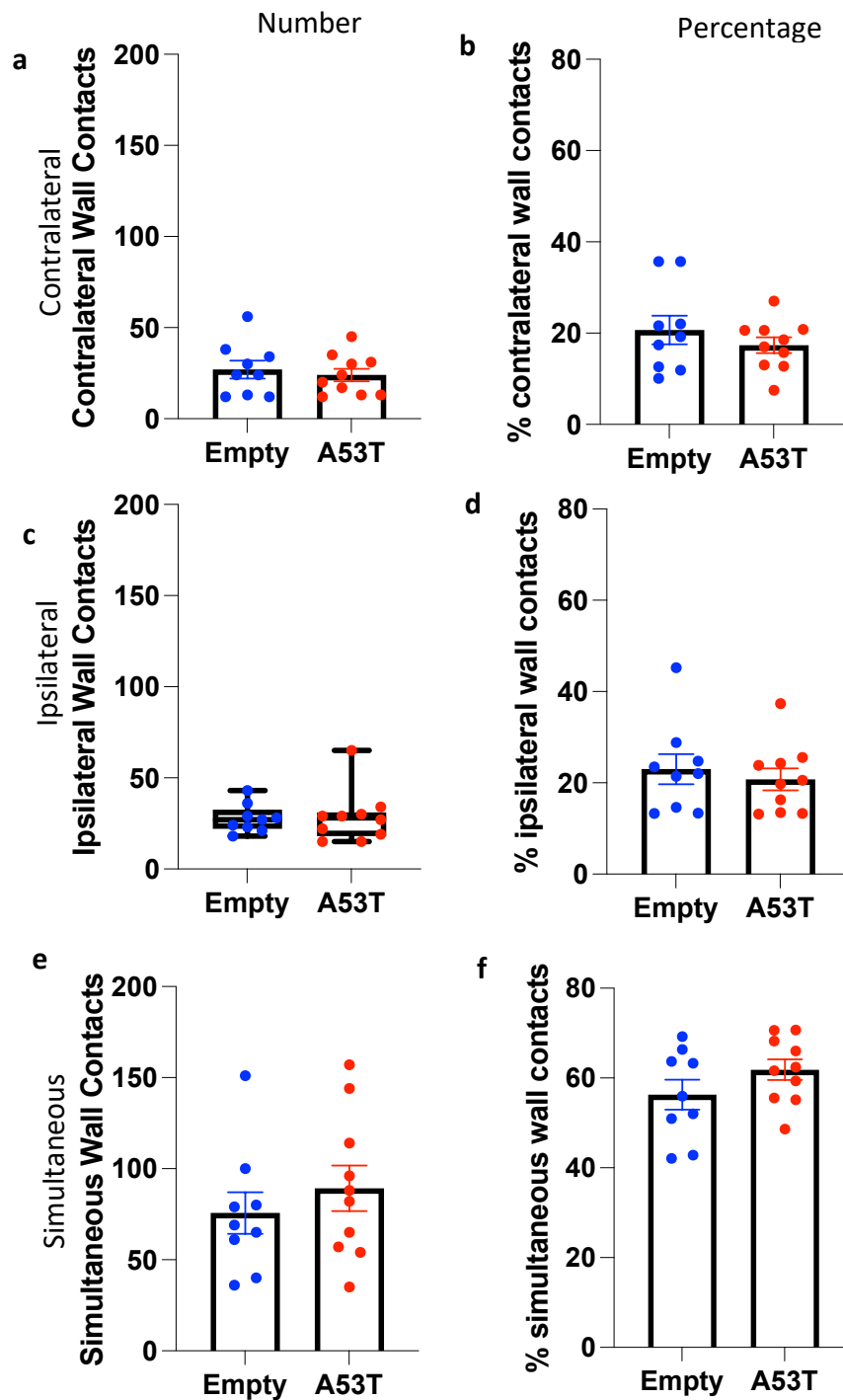


Figure 4.50: Performance in the cylinder test at 86 days post-AAV delivery for females alone. No differences were observed between the treatment groups at 86 days post-AAV delivery in the cylinder test when females were analysed alone. Data presented as mean with SEM (a, b, d, e, f) or median with minimum and maximum (c). Unpaired t-test (a, b, d, e, f) or Mann Whitney test (c). n = 9-10.

4.6.3.11 No differences were identified at 86 days post-AAV delivery in the cylinder test for floor landings when both sexes were combined, or when males were analysed alone.

Analysis of floor landings in the cylinder test at 86 days post-AAV delivery did not identify any differences between the groups when both sexes were analysed together or when males were analysed separately. Data presented in Appendix II.

4.6.3.12 Decreased ipsilateral floor landings were observed when females were analysed alone at 86 days post-AAV delivery.

At 86 days post-AAV delivery, when females were analysed separately, decreased ipsilateral floor landings was observed in the cylinder test. Data presented in Appendix II.

4.6.3.13 Limb use asymmetry was observed in the AAV-A53T females at 86 days post-AAV delivery.

A difference was identified between the groups in limb use asymmetry at 86 days post-AAV delivery when females were analysed alone ($t(17) = 2.207$, $p = 0.0413$), with no differences identified for both sexes combined ($t(37) = 0.0465$, $p = 0.9632$) or when males were analysed alone ($t(18) = 1.717$, $p = 0.1031$) (Figure 4.51).

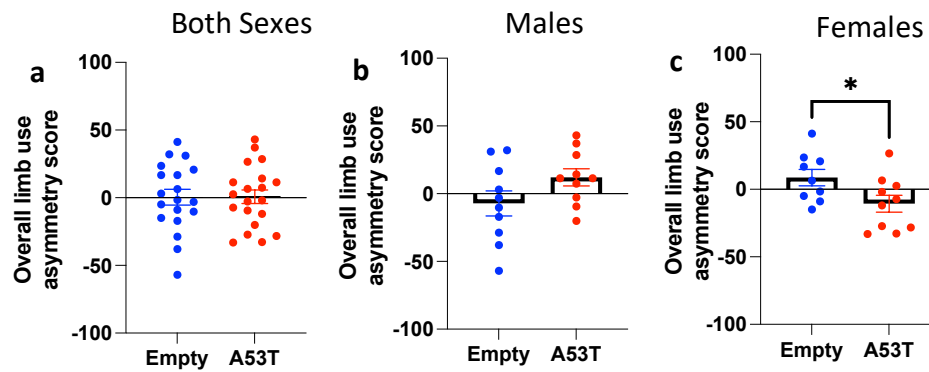


Figure 4.51: Limb use asymmetry 86 days post-AAV delivery in the cylinder test. Decreased limb use asymmetry was observed in the AAV-A53T females when compared with the AAV-empty females (c). Data presented as mean with SEM. Unpaired t-test. $n = 19 - 20$ (a) both sexes combined, $n = 10$ (b) males, $n = 9-10$ (c) females. * $p < 0.05$.

4.6.3.14 No limb use asymmetry was observed for wall alone at 86 days post-AAV delivery.

No differences were identified between the groups in limb use asymmetry for wall at 86 days post-AAV delivery for both sexes combined ($t(37) = 0.7637$, $p = 0.4499$), for males alone ($t(18) = 0.7476$, $p = 0.4644$), or for females alone ($U = 44$, $p = 0.9682$) (Figure 4.52).

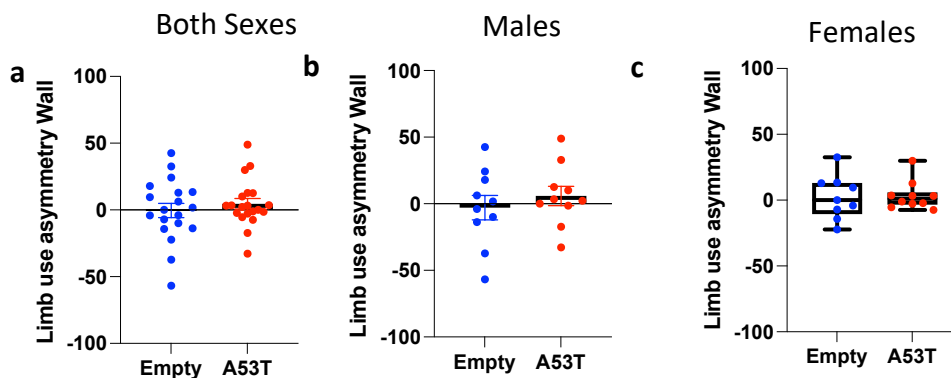


Figure 4.52: Limb use asymmetry 86 days post-AAV delivery in the cylinder test for wall alone. Analysis of limb use asymmetry 86 days post-AAV delivery for wall alone found no differences between the treatment groups. Data presented as mean with SEM (a, b) and median with minimum and maximum (c). Unpaired t-test or Mann Whitney test. $n = 19 - 20$ (a) both sexes combined, $n = 10$ (b) males, $n = 9-10$ (c) females.

4.6.3.15 Limb use asymmetry was observed in the AAV-A53T females at 86 days post-AAV delivery when floor landings were analysed alone.

Analysis of limb use asymmetry at 86 days post-AAV delivery for floor landings found a difference between the groups for females alone ($t(17) = 2.302$, $p = 0.0343$), with no differences identified when both sexes were combined ($t(37) = 0.2917$, $p = 0.7721$) or when males were analysed alone ($t(18) = 1.353$, $p = 0.1928$) (Figure 4.53).

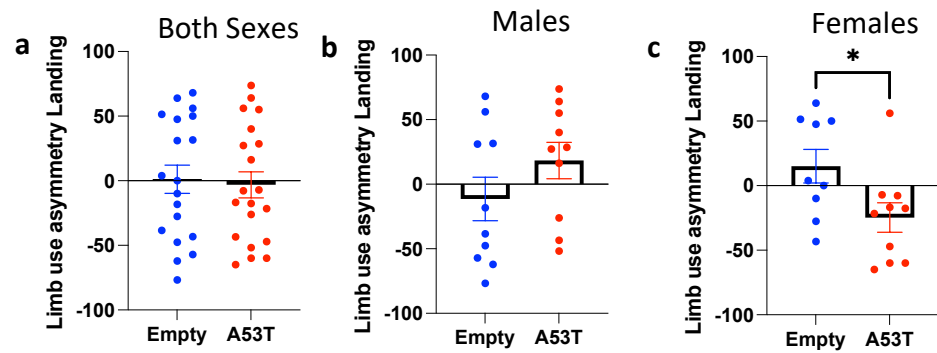


Figure 4.53: Limb use asymmetry 86 days post-AAV delivery in the cylinder test for floor alone. Analysis of limb use asymmetry 86 days post-AAV delivery for floor alone found a difference between the groups when females were analysed alone with the AAV-A53T females having decreased limb use asymmetry for floor landings when compared to the AAV-empty females (c). Data presented as mean with SEM. Unpaired t-test. $n = 19 - 20$ (a) both sexes combined, $n = 10$ (b) males, $n = 9-10$ (c) females. * $p < 0.05$.

4.6.4 Stepping Test

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

This difference in baseline performance in the stepping test is unexpected as the animals should not display any differences pre-AAV delivery. This baseline difference will be taken into consideration when assessing any post-AAV delivery behavioural deficits which may arise for percentage forward steps in the AAV-A53T treatment group. Data presented in Appendix II.

4.6.4.2 Decreased % contralateral forward steps were identified for the AAV-A53T group at baseline when compared with % ipsilateral when males were analysed alone.

This difference in baseline performance in the stepping test is unexpected as the animals should not display any differences pre-AAV delivery. This baseline difference will be taken into consideration when assessing any post-AAV delivery behavioural deficits which may arise for percentage forward steps in the AAV-A53T treatment group. Data presented in Appendix II.

4.6.4.3 Analysis of females alone identified no differences in the stepping test at baseline.

No differences were identified at baseline in the stepping test when females were analysed separately. Data presented in Appendix II.

4.6.4.4 A difference between % contralateral and % ipsilateral forward steps was identified in both the AAV-empty and AAV-A53T groups at 84 days post-AAV delivery when both sexes were analysed together.

The stepping test was repeated at 84 days post-AAV delivery. Analysis of the number of forward steps identified an effect of side (Interaction $F(1, 72) = 0.0644$, $p = 0.8004$; Side $F(1, 72) = 5.635$, $p = 0.0203$; Treatment $F(1, 72) = 0.8937$, $p = 0.3746$). Post-hoc analysis identified no differences between the treatment groups. No effects were identified for number of backward steps (Interaction $F(1, 72) = 0.0470$, $p = 0.8289$; Side $F(1, 72) = 0.3596$, $p = 0.5506$; Treatment $F(1, 72) = 0.3551$, $p = 0.5531$).

An effect of treatment was identified for percentage forward steps (Interaction $F(1, 72) = 0.2005$, $p = 0.6557$; Side $F(1, 72) = 19.48$, $p < 0.0001$; Treatment $F(1, 72) = 0.000$, $p > 0.9999$), with post-hoc comparisons identifying a difference between % contralateral and % ipsilateral in the AAV-A53T group ($p < 0.01$) and the AAV-empty group ($p < 0.01$). As this decreased % contralateral forward stepping was also observed at baseline in the AAV-A53T treatment group this does not necessarily reflect an effect of treatment. No effects were identified for percentage backward steps (Interaction $F(1, 72) = 0.0100$, $p = 0.9206$; Side $F(1, 72) = 1.653$, $p = 0.2026$; Treatment $F(1, 72) = 0.000$, $p > 0.9999$) (Figure 4.54).

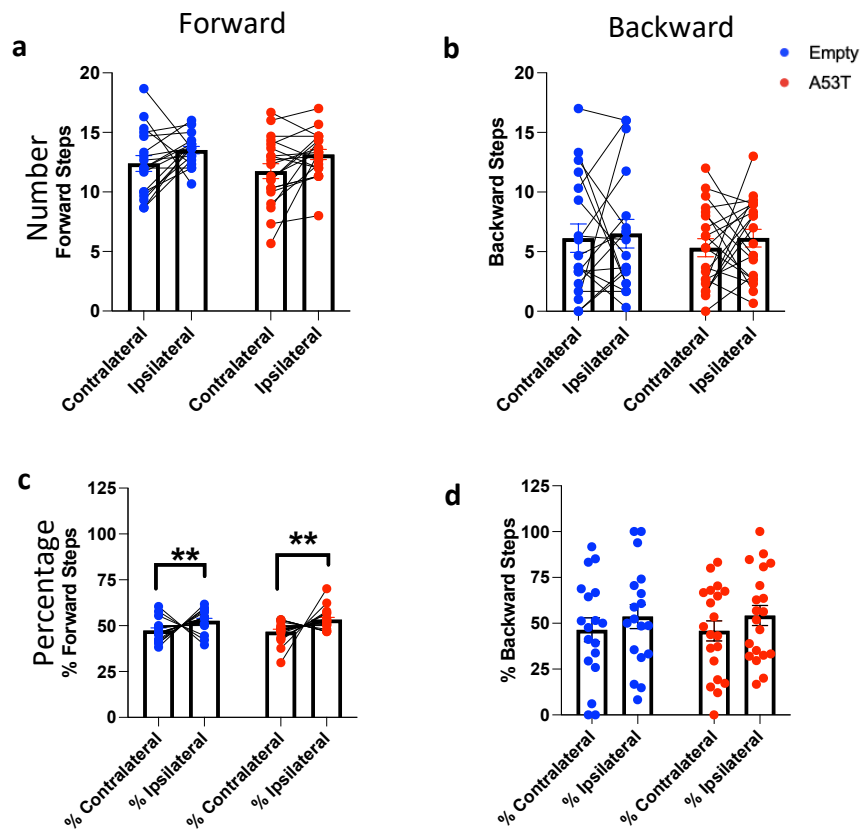


Figure 4.54: Performance in the stepping test at 84 days post-AAV delivery for both sexes combined. Decreased % contralateral forward steps were identified in both the AAV-empty and AAV-A53T groups in the stepping test when compared with their respective % ipsilateral forward steps at 84 days post-AAV delivery. Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons. $n = 18-20$. ** $p < 0.01$.

4.6.4.5 A difference between % contralateral and % ipsilateral forward steps was identified in the AAV-empty group at 84 days post-AAV delivery when males were analysed alone.

Analysis of males alone at 84 days post-AAV delivery found no effects for number of forward steps (Interaction $F(1, 36) = 1.685$, $p = 0.2025$; Side $F(1, 36) = 1.817$, $p = 0.1861$; Treatment $F(1, 36) = 0.4213$, $p = 0.5024$) or backward steps (Interaction $F(1, 36) = 0.4896$, $p = 0.4886$; Side $F(1, 36) = 0.4974$, $p = 0.4852$; Treatment $F(1, 36) = 0.0218$, $p = 0.8834$).

An interaction effect and an effect of side was identified for percentage forward steps (Interaction $F(1, 36) = 7.475$, $p = 0.0096$; Side $F(1, 36) = 10.74$, $p = 0.0023$; Treatment $F(1, 36) = 0.000$, $p > 0.9999$). Post-hoc comparisons identified a difference between the sides for the AAV-empty group ($p < 0.001$). No effects were identified for percentage backward steps (Interaction $F(1, 36) = 2.054$, $p = 0.1604$; Side $F(1, 36) = 2.403$, $p = 0.1298$; Treatment $F(1, 36) = 0.000$, $p > 0.9999$) (Figure 4.55).

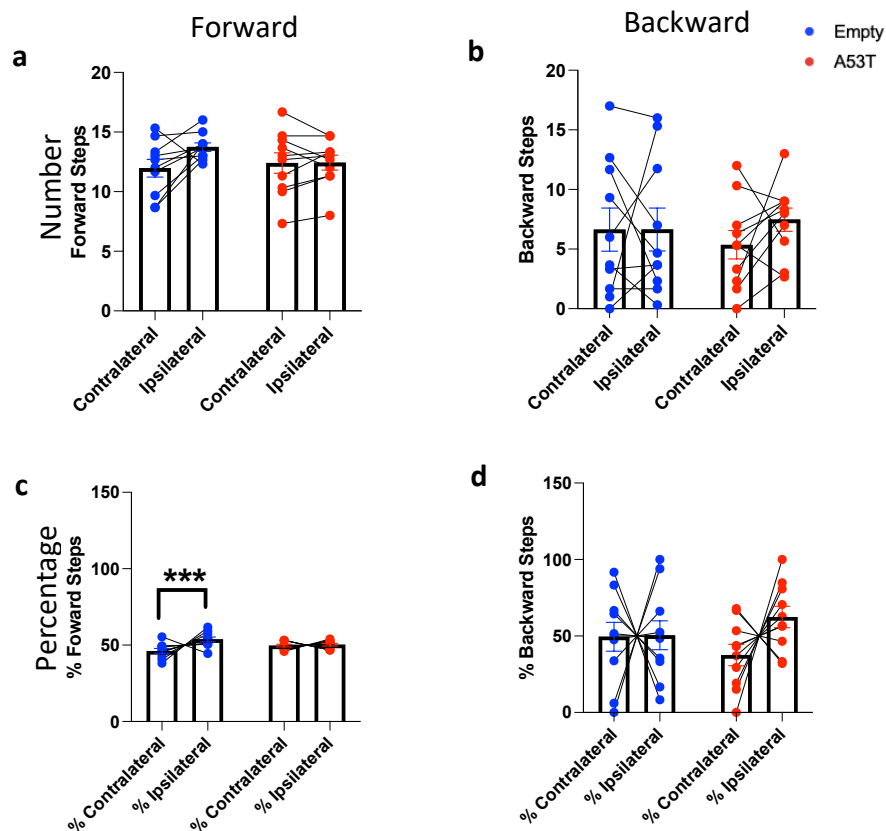


Figure 4.55: Performance in the stepping test for males alone at 84 days post-AAV delivery. Decreased % contralateral forward steps was identified in the AAV-empty males in the stepping test when compared with their respective % ipsilateral forward steps at 84 days post-AAV delivery (c). Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons. $n = 10$. *** $p < 0.001$.

4.6.4.6 A difference between % contralateral and % ipsilateral forward steps was identified in the AAV-A53T group at 84 days post-AAV delivery when females were analysed alone.

Analysis of females alone at 84 days post-AAV delivery found no effects for number of forward steps (Interaction $F(1, 32) = 2.074, p = 0.1596$; Side $F(1, 32) = 3.365, p = 0.0759$; Treatment $F(1, 32) = 0.5043, p = 0.4828$), or backward steps (Interaction $F(1, 32) = 0.2728, p = 0.6051$; Side $F(1, 32) = 0.0171, p = 0.8969$; Treatment $F(1, 32) = 0.4610, p = 0.5021$).

An interaction effect and an effect of side was identified for percentage forward steps (Interaction $F(1, 32) = 4.766, p = 0.0365$; Side $F(1, 32) = 10.54, p = 0.0027$; Treatment $F(1, 32) = 0.000, p > 0.9999$). Post-hoc comparisons identified a difference between % ipsilateral and % contralateral for the AAV-A53T group ($p < 0.01$). No effects were identified for percentage backward steps (Interaction $F(1, 32) = 1.793, p = 0.1900$; Side $F(1, 32) = 0.1464, p = 0.7045$; Treatment ($F(1, 32) = 0.000, p > 0.9999$) (Figure 4.56).

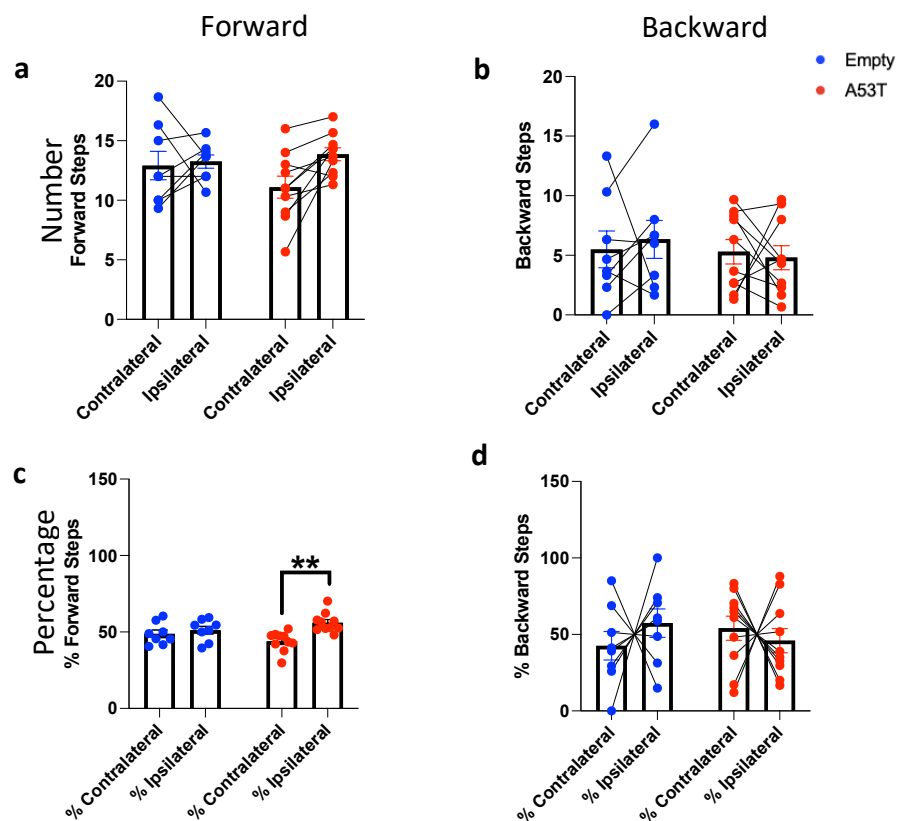


Figure 4.56: Performance in the stepping test for females alone at 84 days post-AAV delivery. Decreased % contralateral forward steps was identified in the AAV-A53T females in the stepping test when compared with their respective % ipsilateral forward steps at 84 days post-AAV delivery (c). Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 8-10.

4.6.5 Amphetamine Rotation Test

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

No differences were identified between the groups for total number of rotations at 22 days post-AAV delivery for both sexes combined ($U = 173$, $p = 0.6415$), for males alone ($U = 43$, $p = 0.6174$), or for females alone ($t(17) = 0.0349$, $p = 0.9726$) (Figure 4.57).

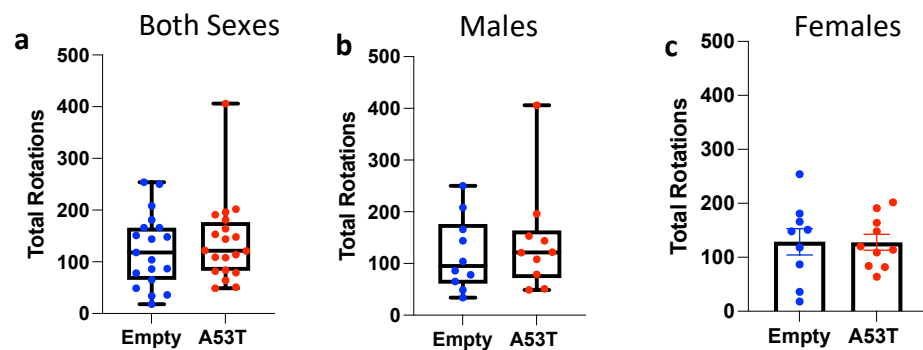


Figure 4.57: Total number of rotations at 22 days post-AAV delivery. No differences were identified at 22 days post-AAV delivery for number of total rotations in the amphetamine rotation test. Data presented as median with minimum and maximum (a, b) or mean with SEM (c). Mann Whitney test (a, b) or unpaired t-test (c). $n = 19 - 20$ (a) both sexes combined, $n = 10$ (b) males, $n = 9 - 10$ (c) females.

4.6.5.2 No differences were identified in the amphetamine rotation test when number of ipsilateral rotations were assessed at 22 days post-AAV delivery.

No differences were identified between the groups at 22 days post-AAV delivery for number of ipsilateral rotations for both sexes combined ($U = 151.5$, $p = 0.2859$), for males alone ($U = 44.5$, $p = 0.6972$), or for females alone ($U = 31$, $p = 0.2682$) (Figure 4.58).

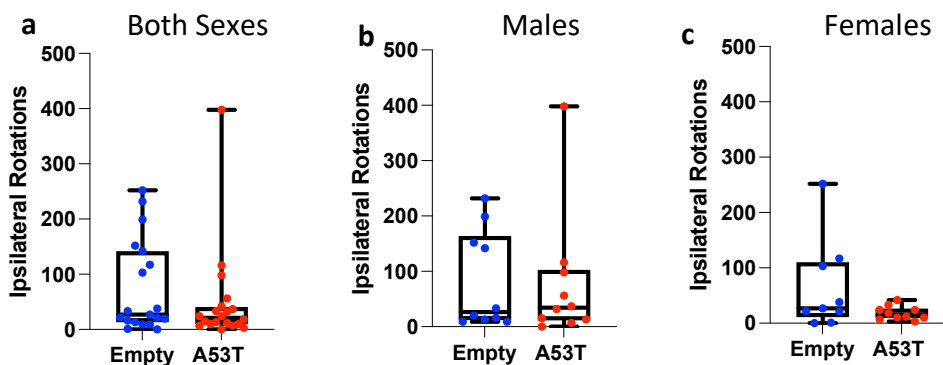


Figure 4.58: Number of ipsilateral rotations at 22 days post-AAV delivery. No differences were identified at 22 days post-AAV delivery in the amphetamine rotation test. Data presented as median with minimum and maximum. Mann Whitney test. $n = 19 - 20$ (a) both sexes combined, $n = 10$ (b) males, $n = 9 - 10$ (c) females.

4.6.5.3 A difference between the groups was identified in the amphetamine rotation test when number of contralateral rotations 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 at 22 days post-AAV delivery when both sexes were analysed together ($U = 116.5$, $p = 0.0385$), with no differences identified when males were analysed alone ($t(18) = 1.292$, $p = 0.2129$) or when females were analysed alone ($t(17) = 1.959$, $p = 0.0667$) (Figure 4.59).

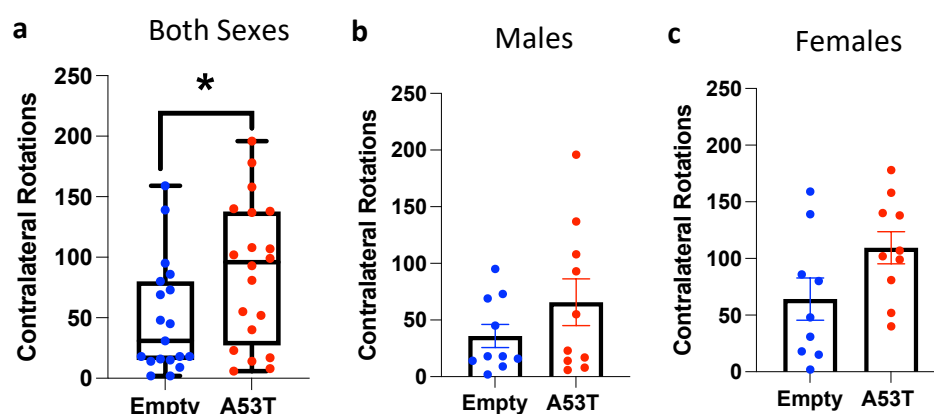


Figure 4.59: Number of contralateral rotations at 22 days post-AAV delivery. Increased contralateral rotations were observed in the amphetamine rotation test in the AAV-A53T group when both sexes were combined (a). Data presented as median with minimum and maximum (a) or mean with SEM (b, c). Mann Whitney test (a) or unpaired t-test (b, c). $n = 19 - 20$ (a) both sexes combined, $n = 10$ (b), males $n = 9 - 10$ (c) females. * $p < 0.05$.

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

Analysis of total rotations per minute at 22 days post-AAV delivery did not identify any differences. Data presented in Appendix II.

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

Analysis of ipsilateral rotations per minute at 22 days post-AAV delivery did not identify any differences. Data presented in Appendix II.

4.6.5.6 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.

Analysis of contralateral rotations per minute at 22 days post-AAV delivery identified a difference when both sexes were combined. Data presented in Appendix II.

4.6.5.7 No differences were identified in the amphetamine rotation test when total number of rotations per minute were assessed at 50-52 days post-AAV delivery.

The amphetamine test was repeated at 50-52 days post-AAV delivery. As the duration of the test was increased over the 50-52 day testing period, it is not possible to express this data as total rotations. The test lengths varied from 60 minutes to 90 minutes and therefore all data will be presented as rotations per minute.

No differences were identified between the groups for total number of rotations per minute at 50-52 days post-AAV delivery for both sexes combined ($U = 177$, $p = 0.9251$), for males alone ($U = 49$, $p = 0.9705$), or for females alone ($t(16) = 0.1940$, $p = 0.8486$) (Figure 4.60).

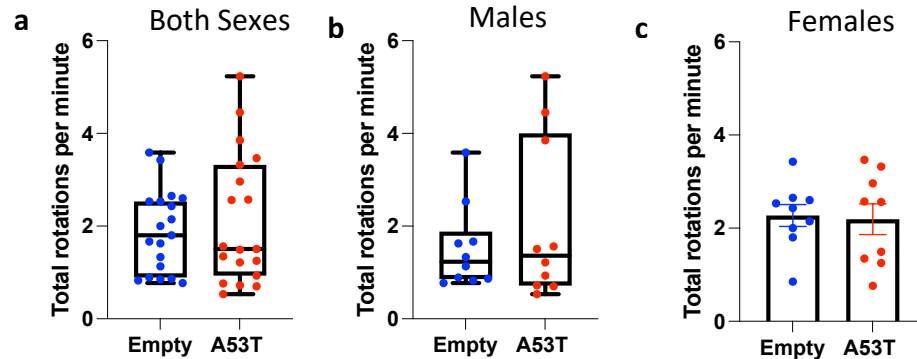


Figure 4.60: Total number of rotations per minute at 50-52 days post-AAV delivery. No differences were identified at 50-52 days post-AAV delivery in the amphetamine rotation test. Data presented as median with minimum and maximum (a, b) or mean with SEM (c). Mann Whitney test (a, b) or unpaired t-test (c). $n = 19 - 20$ (a) both sexes combined, $n = 10$ (b) males, $n = 9 - 10$ (c) females. * $p < 0.05$.

4.6.5.8 Decreased ipsilateral rotations per minute were found for females alone at 50-52 days post-AAV delivery

No differences were identified between the groups for number of ipsilateral rotations per minute at 50-52 days post-AAV delivery for both sexes combined ($U = 131.5$, $p = 0.1562$), or for males alone ($U = 49.5$, $p = 0.9869$). A difference was identified when females were analysed alone ($U = 11$, $p = 0.0076$) (Figure 4.61).

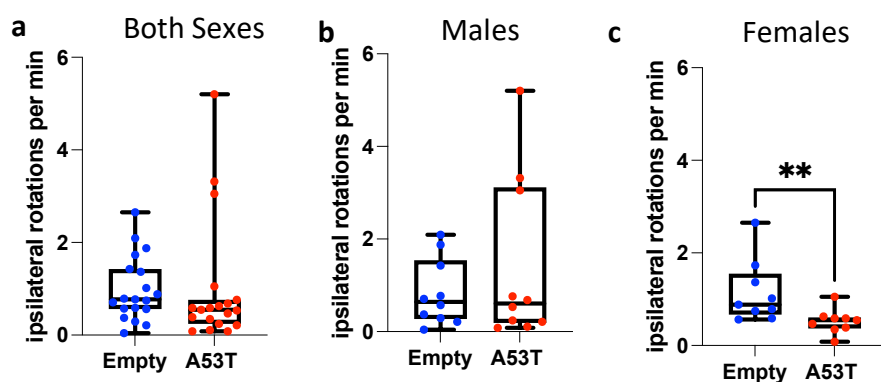


Figure 4.61: Number of ipsilateral rotations per minute at 50-52 days post-AAV delivery. Decreased ipsilateral rotations were observed in the AAV-A53T females in the amphetamine rotation test at 50-52 days post-AAV delivery (c). Data presented as median with minimum and maximum. Mann Whitney test. $n = 19 - 20$ (a) both sexes combined, $n = 10$ (b) males, $n = 9 - 10$ (c) females. ** $p < 0.01$.

4.6.5.9 No differences were identified in the amphetamine rotation test when number of contralateral rotations per minute were assessed at 50-52 days post-AAV delivery.

No differences were identified between the groups for number of contralateral rotations per minute at 50-52 days post-AAV delivery for both sexes combined ($t(36) = 1.024$, $p = 0.3127$), for males alone ($t(18) = 0.1475$, $p = 0.8844$) or for females alone ($t(16) = 1.632$, $p = 0.1222$) (Figure 4.62).

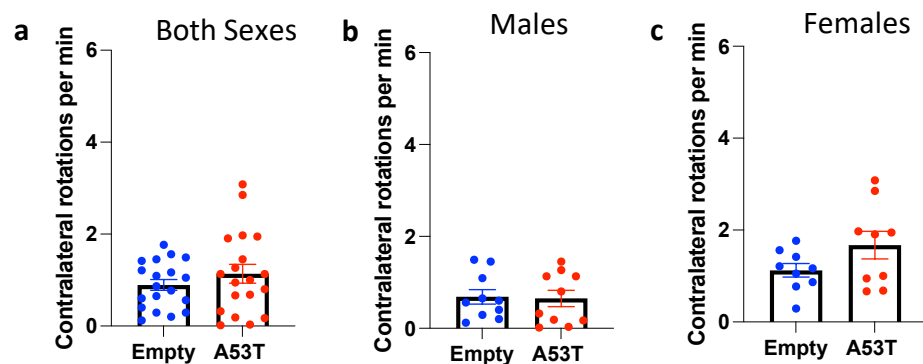


Figure 4.62: Number of contralateral rotations per minute at 50-52 days post-AAV delivery. No differences in contralateral rotations per minute were identified in the amphetamine rotation test at 50-52 days post-AAV delivery. Data presented as mean with SEM. Unpaired t-test. $n = 19 - 20$ (a) both sexes combined, $n = 10$ (b) males, $n = 9 - 10$ (c) females.

4.6.5.10 No differences were identified in the amphetamine rotation test when total number of rotations were assessed at 86 days post-AAV delivery.

No differences were identified between the groups for total number of rotations at 86 days post-AAV delivery for both sexes combined ($U = 186$, $p = 0.9169$), for males alone ($U = 43$, $p = 0.6305$), or for females alone ($t(10.06) = 1.600$, $p = 0.1405$) (Figure 4.63).

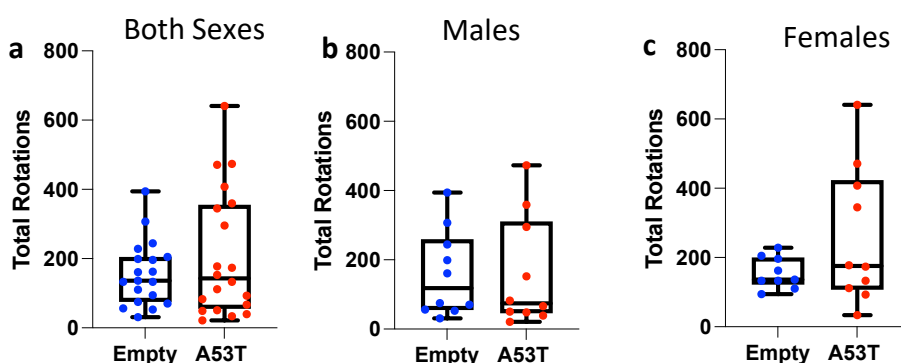


Figure 4.63: Total number of rotations at 86 days post-AAV delivery. No differences were identified at 86 days post-AAV delivery in the amphetamine rotation test when total rotations were analysed. Data presented as median with minimum and maximum. Mann Whitney test (a, b) or unpaired t-test with Welch's correction (c). $n = 19 - 20$ (a) both sexes combined, $n = 10$ (b) males, $n = 9 - 10$ (c) females.

4.6.5.11 No differences were identified in the amphetamine rotation test when the number of ipsilateral rotations were assessed at 86 days post-AAV delivery.

No differences were identified between the groups at 86 days post-AAV delivery for number of ipsilateral rotations for both sexes combined ($U = 168$, $p = 0.5452$), for males alone ($U = 49.5$, $p = 0.9840$), or for females alone ($U = 29$, $p = 0.2032$) (Figure 4.64).

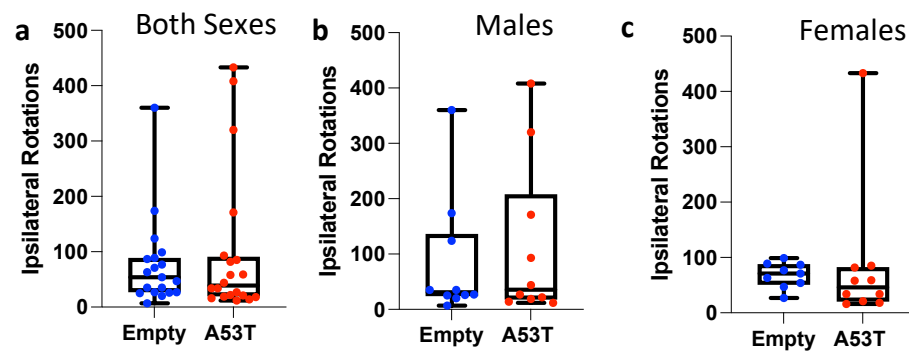


Figure 4.64: Number of ipsilateral rotations at 86 days post-AAV delivery. No differences were identified at 86 days post-AAV delivery in the amphetamine rotation test when ipsilateral rotations were analysed. Data presented as median with minimum and maximum. Mann Whitney test. $n = 19 - 20$ (a) both sexes combined, $n = 10$ (b) males, $n = 9 - 10$ (c) females.

4.6.5.12 No differences were identified in the amphetamine rotation test when the number of contralateral rotations were assessed at 86 days post-AAV delivery.

No differences were identified for number of contralateral rotations at 86 days post-AAV delivery when both sexes were analysed together ($U = 172.5$, $p = 0.6318$), when males were analysed alone ($U = 47$, $p = 0.8534$) or when females were analysed alone ($t(11.29) = 1.885$, $p = 0.0854$) (Figure 4.65).

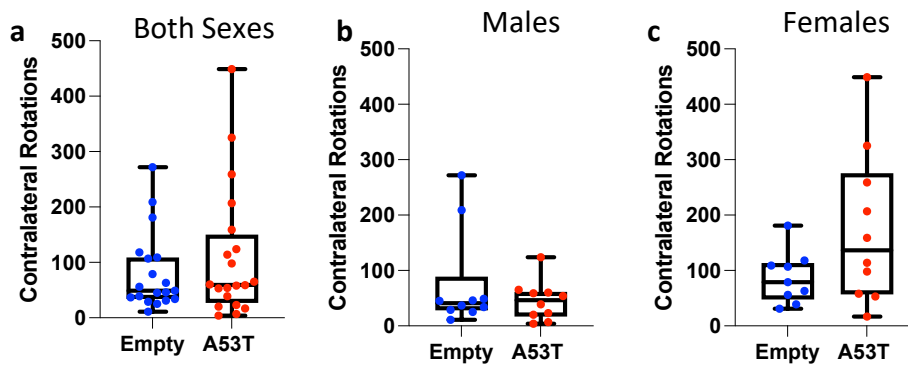


Figure 4.65: Number of contralateral rotations at 86 days post-AAV delivery. No differences were identified at 86 days post-AAV delivery in the amphetamine rotation test when contralateral rotations were analysed. Data presented as median with minimum and maximum. Mann Whitney test (a, b) or unpaired t-test with Welch's correction (c). $n = 19 - 20$ (a) both sexes combined, $n = 10$ (b) males, $n = 9 - 10$ (c) females.

4.6.5.13 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.

Analysis of the number of ipsilateral rotations per minutes identified no differences at 86 days post-AAV delivery. Data presented in Appendix II.

4.6.5.14 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.

Analysis of the number of contralateral rotations per minutes identified no differences at 86 days post-AAV delivery. Data presented in Appendix II.

4.6.6 Tyrosine hydroxylase immunoreactive cells

4.6.6.1 *Decreased TH immunoreactive cells were found in the AAV-A53T injected animals substantia nigra when compared to the AAV-empty injected animals when the data were presented as percentage of the un-injected side at 90 days post-AAV delivery.*

The number of TH immunoreactive 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. A difference was identified between the groups when both sexes were combined ($t(7.197) = 2.963, p = 0.0235$), with no differences identified when males were analysed alone ($t(3.218) = 2.526, p = 0.0801$), or when females were analysed alone ($t(2.121) = 1.571, p = 0.2499$).

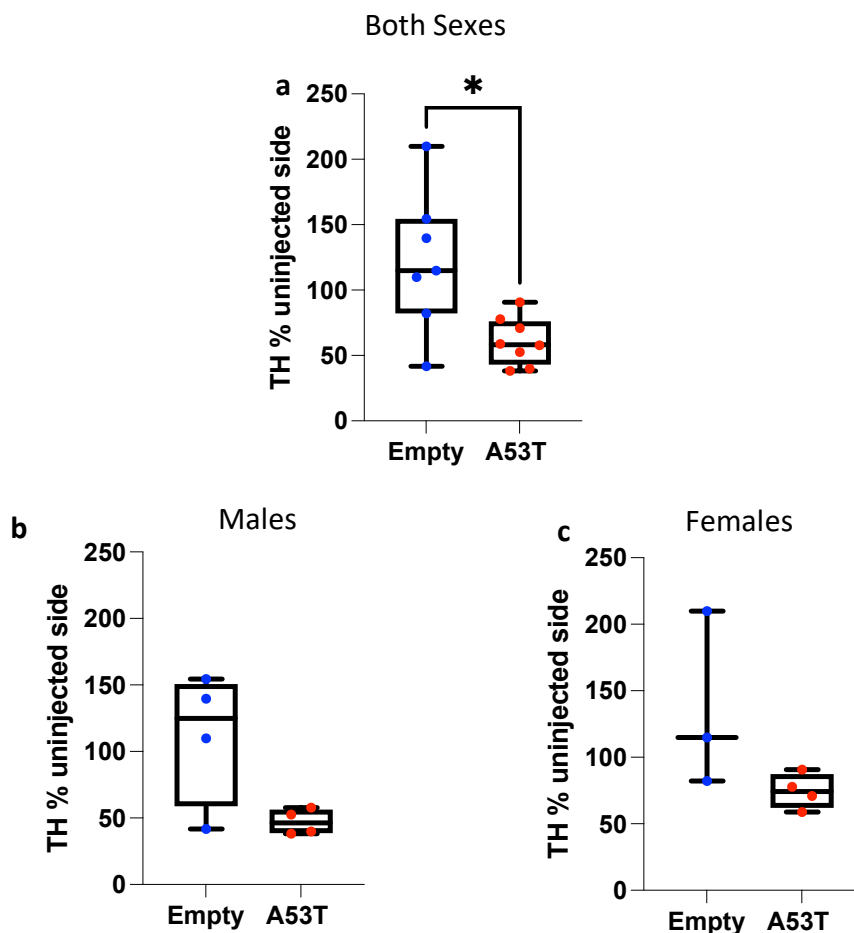


Figure 4.66: Analysis of TH immunoreactive cell counts in the substantia nigra as expressed as a percentage of the un-injected side. Decreased TH immunoreactive cells were observed in the AAV-A53T group when both sexes were analysed together (a). Data presented as median with minimum and maximum. Welch's t test. $n = 7 - 8$ (a) both sexes, $n = 4$ (b) males, $n = 3 - 4$ (c) females. * $p < 0.05$.

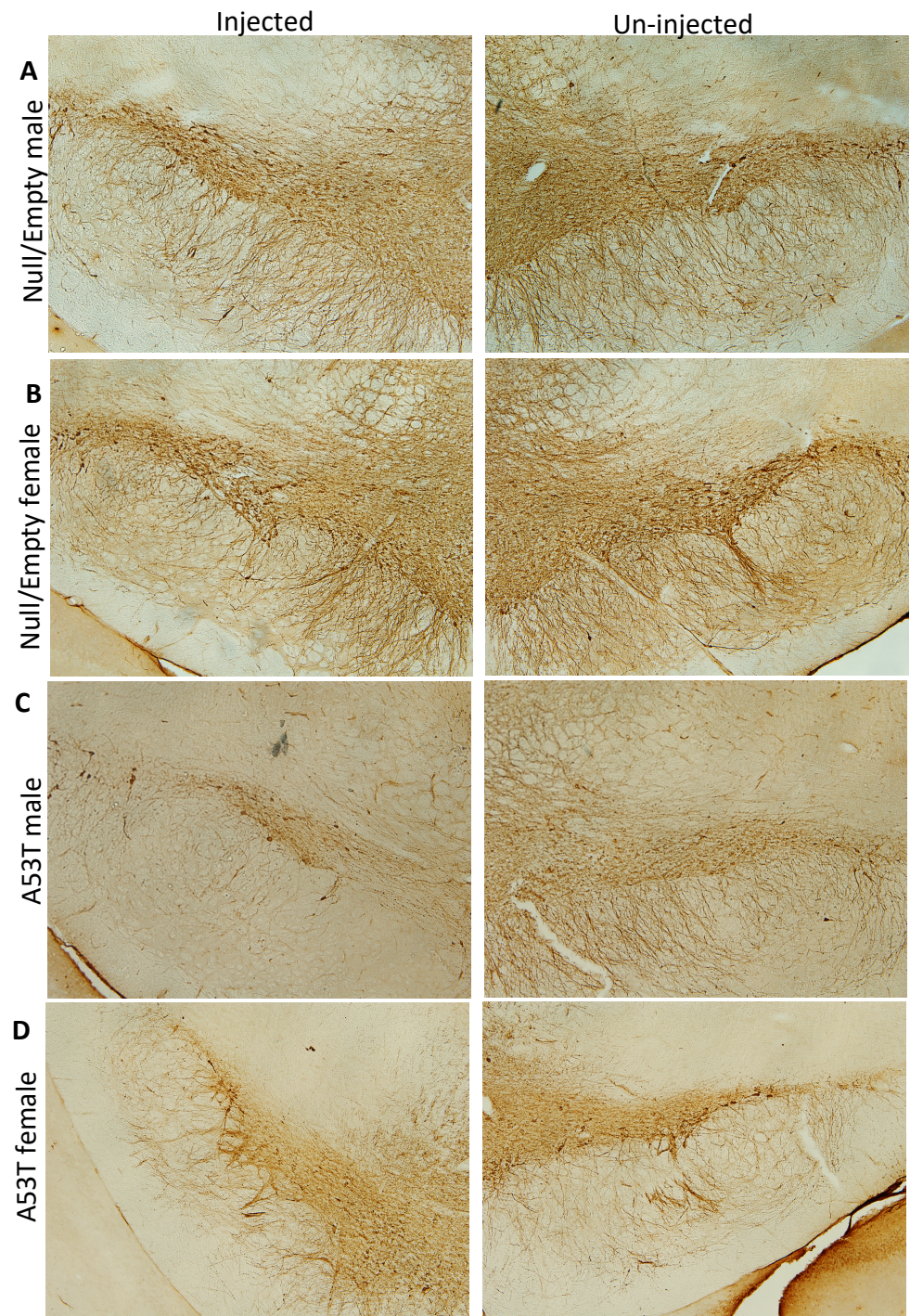


Figure 4.67: Representative images of TH immunoreactivity in the substantia nigra at 13 weeks post-AAV delivery in (a) AAV1/2-CMV-Null/Empty injected male, (b) AAV1/2-CMV-Null/Empty injected female, (c) AAV1/2-CMV-A53T alpha-synuclein injected male, (d) AAV1/2-CMV-A53T alpha-synuclein injected female. Injected hemisphere on left-hand side of panel, un-injected on right. Images acquired at 10X.

4.6.7 Summary of results 13 Week AAV-A53T Study

	Staircase Test multiple time points post- AAV	Cylinder Test 50-52 days post-AAV 86 days post-AAV	Stepping Test 84 days post-AAV	Amphetamine Rotation Test 22 days; 50-52 days; 86 days post-AAV	TH immunoreactivity (% un-injected side) 90 days post-AAV
Both Sexes	No differences	50-52 days: Decreased % contralateral wall contacts in AAV-A53T when compared to AAV-Empty. Increased Limb Use Asymmetry Score Total and Wall in AAV-A53T when compared with AAV- Empty. 86 days: No differences	Decreased % forward contralateral steps in both AAV-empty and AAV- A53T. <i>Interpret with caution for AAV-A53T group as this difference was present at baseline.</i>	22 days: Increased contralateral rotations in AAV- A53T when compared to AAV- Empty. 50-52 days: No differences 86 days: No differences	Decreased in AAV-A53T when compared with AAV-empty.
Male	No differences	50-52 days: Decreased % and number of contralateral wall contacts in AAV-A53T when compared to AAV-Empty. Increased % ipsilateral wall contacts in AAV- A53T when compared to AAV-Empty. Increased Limb Use Asymmetry Score Total Limb Use Asymmetry Score Wall in AAV-A53T when compared with AAV- Empty. 86 days: No differences	Decreased % forward contralateral steps in AAV- empty.	22 days: No differences 50-52 days: No differences 86 days: No differences	No differences
Female	No differences	50-52 days: Increased % simultaneous wall contacts in AAV-A53T when compared with AAV-empty. 86 days: Decreased Limb Use Asymmetry Score Total and Limb Use Asymmetry Score Floor in AAV-A53T when compared with AAV-Empty.	Decreased % forward contralateral steps in AAV- A53T.	22 days: No differences 50-52 days: Decreased ipsilateral rotations per minute in AAV- A53T when compared with AAV-empty. 86 days: No differences	No differences

Table 4.9: A summary of the results obtained in the 13 week AAV-A53T study.

4.7 Discussion

In this study it is shown that unilateral injection of AAV1/2-CMV-A53T-alpha-synuclein leads to alpha-synuclein immunofluorescence in the injected hemisphere of male and female Wistar rats, with immunofluorescence identified at 3.5 weeks post-AAV delivery and continuing to the last time point assessed, 13 weeks post-AAV delivery. This replicates previous studies in which the expression of alpha-synuclein was induced through the use of AAVs which led to the transduction of neurons within the substantia nigra as early as one week post-AAV delivery, with expression continuing up to 27 weeks post-AAV delivery (Kirik et al., 2002, Koprich et al., 2010, Gaugler et al., 2012, Chung et al., 2009).

Behaviourally, impairments were identified at multiple timepoints in the stepping test of forelimb akinesia, however these findings were not always consistent. At 24 days post-AAV delivery, females in the AAV1/2-CMV-A53T-alpha-synuclein group in both the 3.5 week and 6.5 week study showed decreased contralateral stepping when compared to ipsilateral stepping in the backward direction. However, in the 6.5 week study males also showed decreased contralateral stepping in the backward direction when compared to ipsilateral stepping in both the AAV1/2-CMV-A53T-alpha-synuclein and AAV1/2-CMV-Null/Empty groups, although the effect was stronger for the AAV1/2-CMV-A53T-alpha-synuclein group. The stepping test was repeated at 42 days post-AAV delivery, and it was found that this deficit in contralateral backward stepping was evident in the AAV1/2-CMV-A53T-alpha-synuclein group when males and females were analysed together, with analysis of males and females alone not finding any differences. Finally, in the 13 week study, analysis of forward steps found decreased contralateral steps when compared with ipsilateral steps in both the AAV1/2-CMV-A53T-alpha-synuclein and AAV1/2-CMV-Null/Empty groups. Analysis of males alone found decreased contralateral forward steps in the AAV1/2-CMV-Null/Empty, and not AAV1/2-CMV-A53T-alpha-synuclein group, with analysis of females alone finding

decreased contralateral forward steps in the AAV1/2-CMV-A53T-alpha-synuclein group.

Caution must be used when interpreting the stepping test results for both sexes combined and males alone in the AAV1/2-CMV-A53T-alpha-synuclein group as a baseline difference in percentage contralateral forward steps was also observed. Previous research utilising male Sprague Dawley rats has found that post unilateral AAV2/9-A53T alpha-synuclein delivery to the substantia nigra consistent behavioural deficits in the stepping test were observed from four weeks post-delivery up to, and including 18 weeks post-delivery (Becker et al., 2021, Bourdenx et al., 2015). Aside from the impairments observed in the 3.5 and 6.5 week studies in the AAV1/2-CMV-A53T-alpha-synuclein females when contralateral backward steps were assessed, there is little consistency in the results obtained in the present study. The apparent impairment observed in the AAV1/2-CMV-Null/Empty males is concerning in the 13 week study. However, assessment of performance in the stepping test following unilateral AAV-A53T-alpha-synuclein injection into the substantia nigra has not always found deficits. Previous research employing Sprague Dawley rats did not identify any impairments in the stepping test at eight weeks post-AAV A53T alpha-synuclein delivery (Kirik et al., 2002).

Limb use asymmetry was assessed through the cylinder test at multiple timepoints across the three studies. In the 3.5 week study limb use asymmetry was observed in the cylinder test in the AAV1/2-CAG-miR scrambled control alpha-synuclein co-expressing eGFP group, with decreased contralateral limb contacts observed in this group when compared with the PBS injected group. As GFP has been reported to be toxic in previous studies, this result is not unusual (Koprach et al., 2010). Interestingly, assessment of limb use asymmetry at 44 days post-AAV delivery did not identify any differences between the groups in the 6.5 week study. However, exposure to the cylinder test at 50-52 days post-AAV delivery in the 13 week study found decreased percentage contralateral wall contacts in the AAV1/2-CMV-A53T-alpha-synuclein group when both sexes were combined and compared with the AAV1/2-CMV-

Null/Empty group. Assessment of males alone similarly found decreased contralateral wall contacts, when assessed as both raw data and percentage in the AAV1/2-CMV-A53T-alpha-synuclein group when compared with the AAV1/2-CMV-Null/Empty group. Furthermore, increased percentage ipsilateral wall contacts were observed when the AAV1/2-CMV-A53T-alpha-synuclein group was compared to the AAV1/2-CMV-Null/Empty group for males alone, perhaps suggesting some form of compensation.

A deficit in the cylinder test has been observed in multiple studies post-AAV-A53T-alpha-synuclein delivery (Torre-Muruzabal et al., 2019, Crabbe et al., 2019, Van der Perren et al., 2015b, Rodriguez-Perez et al., 2018). Interestingly, in the present study, these deficits were observed in males alone when the sexes were analysed separately, with no deficits in contralateral limb use identified in the females. Previous research in female Wistar rats and female Sprague Dawley rats has identified deficits in the cylinder test following AAV-A53T alpha-synuclein delivery at multiple time points post-AAV delivery which is interesting given the lack of a deficit observed in the females in the present study. Re-assessment in the cylinder test at 84 days post-AAV delivery did not identify any deficits in contralateral limb use. It is possible that the rats had habituated to the environment as they had been exposed to it a month prior. It has also been found that as they age rats exhibit decreased locomotor activity, and due to the extended time-point of this study it is possible that this inhibited their exploratory activity (Gage et al., 1988).

Assessment of fine motor skills in the staircase test across all three studies did not identify any effects of AAV-delivery. Studies utilising AAV vectors to induce expression of alpha-synuclein have also found no effects in the staircase test (Kirik et al., 2002, Van der Perren et al., 2015b). While deficits in the cylinder test were observed following rAAV2/7-mediated overexpression of A53T alpha-synuclein, no deficits were observed in the staircase test (Van der Perren et al., 2015b). The staircase test has identified deficits in other models of Parkinsonism, including models induced by LPS, which produces a robust lesion following intranigral injection, as well as neuroinflammation (O'Neill et al.,

2020, Yssel et al., 2018). It is possible that the effects of AAV-A53T alpha-synuclein expression does not have the same impact as LPS injection, and the staircase test is not the optimal test for the AAV-alpha-synuclein model.

In the 13 week study the amphetamine rotation test was included, in order to assess potential ipsilateral dopaminergic depletion post-AAV delivery. The animals were exposed to the amphetamine rotation test at 22 days post-AAV delivery. Increased total contralateral rotations and contralateral rotations per minute were observed in the AAV1/2-CMV-A53T-alpha-synuclein group when both sexes were combined, with no differences identified when males and females were analysed alone. More detailed assessment of the performance in the amphetamine test identified a sub-group of animals displaying the classical “chasing the tail rotations” associated with amphetamine exposure. Six animals in the AAV1/2-CMV-A53T-alpha-synuclein group and one animal in the AAV1/2-CMV-Null/Empty group displayed this behaviour, with five out of the six animals in the AAV1/2-CMV-A53T-alpha-synuclein group displaying this behaviour in the contralateral and not ipsilateral direction. While this contralateral rotational behaviour is surprising, it has been reported previously in the 6-OHDA model, which is a much more rapid model of neurodegeneration. More specifically, contralateral rotations were observed at four days post-6-OHDA injection into the striatum, even though a 90-95% loss of striatal dopaminergic innervation was observed at this time point (Robinson et al., 1994). It is proposed that this contralateral rotation is due to a transient accumulation of dopamine post-lesion during the degeneration phase, as ipsilateral rotations were then observed at eight days post-6-OHDA lesion.

In the present study at 50-52 days post-AAV delivery, no effects were identified when males were analysed alone, or when both sexes were combined. Analysis of female AAV1/2-CMV-A53T-alpha-synuclein injected rats alone showed decreased ipsilateral rotations per minute when compared with the AAV1/2-CMV-Null/Empty females. This finding is surprising, as increased ipsilateral rotations would be expected if a dopaminergic lesion was present. However, it has been found that the response to amphetamine in females can vary across

their estrous cycle, with greater behaviour responses exhibited on estrous than on other days of the cycle (Becker et al., 2001). It was beyond the scope of the current study to monitor the estrous cycle of the female rats. Future studies should account for the possible impact of the estrous cycle on the behavioural and molecular investigations employed. No differences were identified in the amphetamine rotation test at 86 days post-AAV delivery. It is recommended that at least a month is given between each dose of amphetamine in a longitudinal study, and this was adhered to in the present study (Bjorklund and Dunnett, 2019). From the results obtained it would appear that the intra-nigral injection of AAV1/2-CMV-A53T-alpha-synuclein does not lead to a large enough dopaminergic lesion to induce ipsilateral rotations in the amphetamine rotation test.

Early TH cell loss was observed in both the animals injected with AAV1/2-CMV-A53T-alpha-synuclein and AAV1/2-CAG-miR scrambled control alpha-synuclein co-expressing eGFP at 3.5 weeks post-AAV delivery when compared with PBS injected animals. Toxicity of GFP has been reported in previous research (Koprach et al., 2010). While the inclusion of an AAV which leads to the expression of another protein is a useful tool in order to eliminate the impact of the expression of a foreign protein as the reason for the degeneration observed, it was decided that for the 6.5 and 13 week studies the GFP group would be removed. While this is a limiting factor of the longer term studies discussed, and does lead to questions around whether any behavioural changes observed were truly due to the A53T alpha-synuclein, and not just due to the presence of a foreign protein, many studies use an empty AAV vector or the un-injected hemisphere as control and do not account for the expression of a foreign protein (Febbraro et al., 2013, Thakur et al., 2017, Musacchio et al., 2017, Schneider et al., 2019, Sanchez-Guajardo et al., 2013). When assessed at 6.5 weeks post-AAV delivery, decreased TH positive cells were also observed in the AAV1/2-CMV-A53T-alpha-synuclein group when expressed as a percentage of the un-injected side. Due to the lack of behavioural differences observed between males and females, with the deficit in the stepping test apparent for both sexes, TH cell loss was not compared for males and females separately.

Finally, decreased number of TH immunoreactive cells were also observed when the data were analysed as percentage of the un-injected side at 90 days post-AAV delivery in the AAV1/2-CMV-A53T-alpha-synuclein injected rats when both sexes were analysed together. Analysis of males and females separately did not identify any differences, however the number of tissue samples available to analyse did not allow a full picture to be created. This was due to some issues during the processing of the tissue.

Taken together, the results of the above time course study show that AAV1/2-CMV-A53T-alpha-synuclein is effective at leading to the overexpression of alpha-synuclein following unilateral intranigral injection. Furthermore, this expression leads to decreased TH positive immunoreactivity as early as 3.5 weeks post-delivery, and this decreased immunoreactivity is also observed at 6.5 and 13 weeks post-AAV delivery. Behaviourally, there appears to be sex differences in the results obtained, suggesting that either different motor behavioural tests are more applicable to one sex over the other, or that the impact of the delivery of AAV1/2-CMV-A53T-alpha-synuclein is different between males and females. More research is needed in this area, as most studies to date have used either males or females, with no comparative studies of the sexes in relation to the impact of intranigral AAV1/2-CMV-A53T-alpha-synuclein in Wistar rats. Many studies utilising AAV-mediated delivery of A53T alpha-synuclein have performed the studies over much longer time courses post-delivery. While this may lead to more consistent behavioural results it was beyond the scope of the present study which aimed to develop a model with a more rapid onset of behavioural symptoms.