"there appears to be sufficient reason for hoping that some remedial process may ere long be discovered."

James Parkinson, 1817.
THE EFFECT OF CHRONIC L-DOPA TREATMENT ON THE SURVIVAL
AND FUNCTION OF FOETAL VENTRAL MESENCEPHALIC GRAFTS IN
RATS WITH A UNILATERAL 6-OHDA LESION

STAVIA BRIGITTE BLUNT

SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
UNIVERSITY OF LONDON

Department of Clinical Neurology,
Institute of Neurology,
Queen Square,
London WC1N 3BG.
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The effect of chronic treatment with L-3,4-dihydroxyphenylalanine (L-DOPA) and α-methyl-dopahydr azine (carbidopa), at target doses of 200mg/kg/24h and 25mg/kg/24h respectively, on the survival and function of rat foetal ventral mesencephalic (VM) grafts in rats with a unilateral 6-hydroxydopamine (6-OHDA) lesion of the nigrostriatal pathway was assessed.

Animals receiving foetal VM grafts showed a reduction of apomorphine-induced contralateral rotation, and abolition of (+)-amphetamine-induced ipsilateral rotation. This effect of the grafts was not altered by 5 weeks' treatment with L-DOPA and carbidopa. The foetal VM grafts prevented development of marked apomorphine-induced stereotypy which was otherwise seen in lesioned rats receiving sham grafts and L-DOPA treatment. Dopamine cell numbers and their morphology (assessed with tyrosine hydroxylase immunohistochemistry) and fibre outgrowth (assessed with 3H-mazindol autoradiography) were not affected by this treatment.

 Autoradiographic studies of specific 3H-spiperone and 3H-SCH 23390 binding to striatal slices demonstrated that a 6-OHDA lesion increased specific 3H-spiperone binding ipsilaterally, most marked in the lateral and dorsomedial quadrants of the mid-body of the striatum (Level 2). Specific 3H-SCH 23390 binding was inconsistently affected by the lesion apart from an ipsilateral decrease in the ventro-lateral quadrant of the mid-body of the striatum (Level 2). The lesion-induced changes in specific 3H-spiperone binding were reversed by foetal VM grafts, but not by L-DOPA and carbidopa treatment for 5 weeks. Specific 3H-SCH 23390 binding density was not affected by either foetal grafts or by L-DOPA treatment, or a combination of the two.

L-DOPA and carbidopa treatment for 27 weeks of animals receiving foetal grafts did not interfere with
the grafts' abilities to sustain abolition of (+)-amphetamine-induced ipsilateral rotation, and reduction of apomorphine-induced contralateral rotation. Dopamine cell survival in these grafts and their morphology were not affected by this treatment. However, within the grafts of animals treated with L-DOPA and carbidopa, the number of reactive astrocytes was increased. Also, L-DOPA treatment for 27 weeks resulted in a further reduction in the number of dopamine cells that remained in the ventral tegmental area ipsilateral to the 6-OHDA lesion.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>10</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>13</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>17</td>
</tr>
<tr>
<td>PUBLICATIONS RESULTING FROM THIS THESIS</td>
<td>18</td>
</tr>
</tbody>
</table>

CHAPTER 1. GENERAL INTRODUCTION 19

1.1. PARKINSON'S DISEASE 20
1.1.1. Definition, clinical features and diagnosis 20
1.1.2. Epidemiology and Aetiology 20
1.1.3. Anatomy of Parkinson's disease 23
1.1.4. Pathology of Parkinson's disease 24
1.1.5. Biochemistry of Parkinson's disease 27
1.1.6. Dopamine receptor changes in Parkinson's disease 29
1.1.7. Drug Treatment of Parkinson's disease 29
1.1.7.1. Drugs commonly used 29
1.1.7.2. Treatment with L-DOPA 30
1.1.8. Neural Transplantation - A novel approach to treatment 34

1.2. ANIMAL MODELS OF PARKINSON'S DISEASE 34
1.2.1. A rat model of Parkinson's disease 35
1.2.2. The MPTP primate model of Parkinson's disease 37

1.3. ALTERNATIVE TREATMENT FOR PARKINSON'S DISEASE: NEURAL AND ADRENAL MEDULLA TRANSPLANTATION 38
1.3.1. Adrenal medulla transplants in animals 39
1.3.1.1. Studies in 6-OHDA lesioned rats and MPTP-treated mice 39
1.3.1.2. Studies in non-human primates 40
1.3.2. Adrenal medulla transplants in man 41
1.3.3. Neural transplantation in experimental animals 43
1.3.3.1. History 43
1.3.3.2. Studies of rat foetal ventral mesencephalic grafts in 6-OHDA lesioned rats 43
1.3.3.3. Studies of human foetal ventral mesencephalic grafts in 6-OHDA lesioned rats 48
1.3.3.4. Foetal ventral mesencephalic grafts in non-human primates 49
1.3.4. Foetal ventral mesencephalic transplants in man 50
  1.3.4.1. Summary of reported clinical experience 50
  1.3.4.2. Unresolved issues 52

1.4. AIM OF THESIS 57

CHAPTER 2. MATERIALS AND METHODS 58
2.1. INTRODUCTION 59
2.2. DRUGS AND CHEMICALS 60
2.3. SURGICAL TECHNIQUES 60
  2.3.1. Unilateral 6-OHDA-stereotaxic lesion of the medial forebrain bundle 60
  2.3.2. Preparation of rat foetal ventral mesencephalic cell suspensions 61
    2.3.2.1. Timed pregnancies 61
    2.3.2.2. Dissection of ventral mesencephalon and preparation of cell suspensions 65
    2.3.2.3. Assessment of cell viability and concentration in suspensions 68
  2.3.3. Stereotaxic implantation surgery 70

2.4. BEHAVIOURAL TESTS USED IN THE ASSESSMENT OF THE 6-OHDA LESION, FOETAL VENTRAL MESENCEPHALIC GRAFT AND L-DOPA TREATMENT 71
  2.4.1. Spontaneous rotation 71
  2.4.2. Apomorphine-induced rotation and stereotypy 71
  2.4.3. (+)-Amphetamine induced rotation 74

2.5. L-DOPA AND CARBIDOPA TREATMENT 74

2.6. HISTOLOGICAL AND IMMUNOHISTOCHEMICAL PROCEDURES 77
  2.6.1. Preparation of brains 77
  2.6.2. Slide subbing 78
  2.6.3. Cresyl violet staining 78
  2.6.4. Immunohistochemistry 79
### Contents contd.

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6.5. Cell counting and graft volume estimation</td>
<td>81</td>
</tr>
<tr>
<td>2.6.5.1. Tyrosine hydroxylase stained sections</td>
<td>81</td>
</tr>
<tr>
<td>2.6.5.2. Glial fibrillary acidic protein stained sections</td>
<td>81</td>
</tr>
<tr>
<td>2.7. BIOCHEMICAL ASSESSMENT—HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC MEASUREMENT OF L-DOPA AND ITS METABOLITES IN STRIATUM AND PLASMA</td>
<td>83</td>
</tr>
<tr>
<td>2.7.1. Tissue preparation</td>
<td>83</td>
</tr>
<tr>
<td>2.7.2. HPLC measurement of L-DOPA and its metabolites using electrochemical detection</td>
<td>84</td>
</tr>
<tr>
<td>2.7.3. Calibration of the HPLC system</td>
<td>85</td>
</tr>
<tr>
<td>2.7.4. Recovery from tissue homogenates</td>
<td>88</td>
</tr>
<tr>
<td>2.7.5. Calculation of results</td>
<td>88</td>
</tr>
<tr>
<td>2.8. AUTORADIOGRAPHY OF (^3)H-MAZINDOL, (^3)H-SPERONE and (^3)H-SCH 23390 TO STRIATAL SLICES.</td>
<td>93</td>
</tr>
<tr>
<td>2.8.1. Tissue preparation</td>
<td>93</td>
</tr>
<tr>
<td>2.8.2. Preliminary biochemical studies for (^3)H-spiperone binding to striatal slices</td>
<td>93</td>
</tr>
<tr>
<td>2.8.3. Preliminary biochemical studies for (^3)H-SCH 23390 binding to striatal slices</td>
<td>94</td>
</tr>
<tr>
<td>2.8.4. Scintillation counting of radioactivity bound to striatal slices</td>
<td>99</td>
</tr>
<tr>
<td>2.8.5. Calculation of results</td>
<td>104</td>
</tr>
<tr>
<td>2.8.6. Graphical analysis of preliminary binding data</td>
<td>105</td>
</tr>
<tr>
<td>2.8.7. In Vitro slice binding for autoradiography</td>
<td>105</td>
</tr>
<tr>
<td>2.8.8. Exposure and development of films</td>
<td>106</td>
</tr>
<tr>
<td>2.8.9. Computer-assisted densitometric analysis of autoradiograms</td>
<td>107</td>
</tr>
<tr>
<td>2.9. STATISTICAL ANALYSIS</td>
<td>110</td>
</tr>
<tr>
<td>2.9.1. Apomorphine- and (+)-amphetamine-induced behaviour</td>
<td>110</td>
</tr>
<tr>
<td>2.9.2. Fluid intake and body weights</td>
<td>110</td>
</tr>
<tr>
<td>2.9.3. Histological data</td>
<td>110</td>
</tr>
<tr>
<td>2.9.4. Autoradiographical studies</td>
<td>110</td>
</tr>
<tr>
<td>Contents contd.</td>
<td>PAGE</td>
</tr>
<tr>
<td>---------------</td>
<td>------</td>
</tr>
<tr>
<td>2.10. EXPERIMENTAL DESIGN</td>
<td>111</td>
</tr>
</tbody>
</table>

RESULTS

CHAPTER 3. EFFECTS OF TREATMENT WITH L-DOPA AND CARBIDOPA ADMINISTERED IN THE DRINKING WATER ON MOTOR FUNCTION, BODY WEIGHT AND FLUID INTAKE IN 6-OHDA-LESIONED RATS RECEIVING FOETAL VENTRAL MESENCEPHALIC GRAFTS | 114 |
| 3.1. INTRODUCTION | 115 |
| 3.2. METHODS | 116 |
| 3.3. RESULTS | 119 |
| 3.3.1. Assessment of the lesion | 119 |
| 3.3.2. Fluid intake, body weight and drug treatment | 120 |
| 3.3.3. Motor behaviour | 124 |
| 3.4. DISCUSSION | 131 |

CHAPTER 4. THE EFFECTS OF CHRONIC TREATMENT WITH L-DOPA AND CARBIDOPA ON THE SURVIVAL OF RAT FOETAL VENTRAL MESENCEPHALIC GRAFTS ASSESSED BY TYROSINE HYDROXYLASE IMMUNOHISTOCHEMISTRY AND $^3$H-MAZINDOL AUTORADIOGRAPHY. | 136 |
| 4.1. INTRODUCTION | 137 |
| 4.2. METHODS | 138 |
| 4.3. RESULTS | 142 |
| 4.3.1. Behavioural assessment | 142 |
| 4.3.2. TH immunohistochemical study of midbrain and striatum | 145 |
| 4.3.3. $^3$H-mazindol autoradiography | 153 |
| 4.4. DISCUSSION | 159 |

CHAPTER 5. EFFECTS OF A UNILATERAL 6-OHDA LESION, CHRONIC L-DOPA AND CARBIDOPA TREATMENT AND FOETAL VENTRAL MESENCEPHALIC GRAFTS ON THE DENSITIES OF D-1 AND D-2 DOPAMINE RECEPTORS IN STRIATAL SLICES AS REVEALED BY $^3$H-SCH 23390 AND $^3$H-SPIPERONE AUTORADIOGRAPHY | 164 |
| 5.1. INTRODUCTION | 165 |
| 5.2. METHODS | 166 |
CHAPTER 6. EFFECTS OF LONG-TERM TREATMENT (27 WEEKS) WITH L-DOPA AND CARBIDOPA ON MOTOR FUNCTION, SURVIVAL OF FOETAL VENTRAL MESENCEPHALIC GRAFTS AND GLIOSIS IN 6-OHDA-LESIONED RATS.

6.1. INTRODUCTION

6.2. METHODS

6.3. RESULTS

6.3.1. Drug intake and body weight

6.3.2. Rotational behaviour

6.3.3. Tyrosine hydroxylase immunohistochemistry

6.3.4. GFAP immunohistochemistry

6.4. DISCUSSION

CHAPTER 7. GENERAL DISCUSSION

7.1. SUMMARY OF FINDINGS

7.2. SUITABILITY OF THE 6-OHDA LESION MODEL OF PARKINSON'S DISEASE FOR THE STUDY OF FOETAL GRAFTS

7.3. VIABILITY OF GRAFTS IMPLANTED INTO THE 6-OHDA LESIONED STRIATUM

7.4. SPECIES, NEUROMELANIN AND AGE DIFFERENCES AFFECTING SUSCEPTIBILITY TO TOXIC SUBSTANCES

7.5. L-DOPA TREATMENT REGIME

7.6. FUTURE DIRECTIONS

7.7. SUMMARY

REFERENCES
LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Adrenal medulla transplants in man.</td>
<td>42</td>
</tr>
<tr>
<td>1.2. Foetal ventral mesencephalic transplants in man.</td>
<td>51</td>
</tr>
<tr>
<td>2.1. Cellular constituents of vaginal smears of sexually mature female rats.</td>
<td>66</td>
</tr>
<tr>
<td>2.2. Protocol for cresyl violet staining.</td>
<td>80</td>
</tr>
<tr>
<td>3.1. The proportion of 6-OHDA lesioned rats showing stereotypy following administration of apomorphine (0.5mg/kg sc) before and after a sham graft (groups A and B) or a foetal graft (groups C and D), with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment.</td>
<td>130</td>
</tr>
<tr>
<td>4.1. Rotational rates of 6-OHDA lesioned rats following administration of apomorphine (0.5mg/kg sc) before and after sham graft (groups A and B) or foetal graft (groups C and D) either alone (groups A and C) or following treatment with L-DOPA and carbidopa (gps. B and D) for those rats subsequently examined using TH immunohistochemistry (A) or ^3H^-mazindol binding to striatal slices (B).</td>
<td>143</td>
</tr>
<tr>
<td>4.2. Rotational rates of 6-OHDA lesioned rats following administration of (+)-amphetamine before and after sham graft (groups A and B) or foetal graft (groups C and D), either alone (groups A and C), or following treatment with L-DOPA and carbidopa (groups B and D) for those animals subsequently examined using TH immunohistochemistry (A) or ^3H^-mazindol binding to striatal slices (B).</td>
<td>144</td>
</tr>
<tr>
<td>4.3. The mean number of TH-positive cells in the SN and VTA in sections taken through the ventral mesencephalon of animals with a unilateral 6-OHDA lesion receiving foetal grafts alone (group C) or followed by L-DOPA and carbidopa treatment (group D).</td>
<td>146</td>
</tr>
<tr>
<td>4.4. The density of specific ^3H^-mazindol binding at Level 1 in the intact and 6-OHDA lesioned striatum of animals receiving sham grafts (groups A and B) or foetal grafts (groups C and D) either</td>
<td>10</td>
</tr>
</tbody>
</table>
4.5. The density of specific $^3$H-mazindol binding at Level 2 in the intact and 6-OHDA lesioned striatum of animals receiving a sham graft (groups A and B) or foetal graft (groups C and D) either with (groups B and D) or without (groups A and C) treatment with L-DOPA and carbidopa.

5.1. Rotational rates of 6-OHDA lesioned rats following administration of apomorphine (0.5mg/kg sc) before and after a sham graft (groups A and B) or a foetal graft (groups C and D) either alone (groups A and C) or following treatment with L-DOPA and carbidopa (groups B and D).

5.2. Rotational rates of 6-OHDA lesioned rats following administration of (+)-amphetamine before and after a sham graft (groups A and B) or a foetal graft (groups C and D) either alone (groups A and C) or following treatment with L-DOPA and carbidopa (groups B and D).

5.3. The autoradiographic density of specific $^3$H-spiperone binding to striatal slices taken through Level 1 in the intact and 6-OHDA lesioned striatum of animals receiving a sham graft (groups A and B) or a foetal graft (groups C and D) either with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment.

5.4. The autoradiographic density of specific $^3$H-spiperone binding to striatal slices taken through Level 2 in the intact and 6-OHDA lesioned striatum of animals receiving a sham graft (groups A and B) or a foetal graft (groups C and D) either with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment.

5.5. The autoradiographic density of specific $^3$H-spiperone binding to striatal slices taken through Level 3 in the intact and 6-OHDA lesioned striatum
5.6. Summary of the direction of change in specific \(^3\)H-spiperone binding in the lesioned striatum compared with the intact side. 183

5.7. The autoradiographic density of specific \(^3\)H-SCH 23390 binding to striatal slices taken through Level 1 in the intact and 6-OHDA lesioned striatum of animals receiving a sham graft (groups A and B) or a foetal graft (groups C and D) either with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment. 187

5.8. The autoradiographic density of specific \(^3\)H-SCH 23390 binding to striatal slices taken through Level 2 in the intact and 6-OHDA lesioned striatum of animals receiving a sham graft (groups A and B) or a foetal graft (groups C and D) either with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment. 188

5.9. The autoradiographic density of specific \(^3\)H-SCH 23390 binding to striatal slices taken through Level 3 in the intact and 6-OHDA lesioned striatum of animals receiving a sham graft (groups A and B) or a foetal graft (groups C and D) either with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment. 189

5.10. Summary of the direction of change in specific \(^3\)H-SCH 23390 binding in the lesioned striatum compared with the intact side. 193

6.1. Apomorphine-induced contralateral rotation rates.216

6.2. TH-positive cell number in sections through SN and VTA of the intact and lesioned mesencephalon.218

6.3. Glial cells in intact and lesioned striatum. 220
<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Basal ganglia connections and transmitter systems.</td>
<td>25</td>
</tr>
<tr>
<td>1.2 The neurotoxic species produced in the oxidation of DOPA and dopamine.</td>
<td>54</td>
</tr>
<tr>
<td>2.1 Tyrosine hydroxylase staining of a section through the intact (A) and 6-OHDA lesioned (B) SN, and through the intact and 6-OHDA lesioned VTA (C).</td>
<td>62</td>
</tr>
<tr>
<td>2.2 Tyrosine hydroxylase staining of a section through the intact side of the midbrain through level AP -5.8 from bregma (Paxinos and Watson 1982).</td>
<td>64</td>
</tr>
<tr>
<td>2.3 A diagram of the rat foetal brain as seen under the operating microscope, illustrating important landmarks.</td>
<td>67</td>
</tr>
<tr>
<td>2.4 Vital staining with acridine orange and ethidium bromide of a cell suspension prepared from rat foetal ventral mesencephalon.</td>
<td>69</td>
</tr>
<tr>
<td>2.5 Apomorphine-HCl dose-response curve (A) and time course (B) of contralateral rotational response following administration of apomorphine-HCl to rats with a unilateral 6-OHDA lesion.</td>
<td>72</td>
</tr>
<tr>
<td>2.6 (+)-Amphetamine sulphate dose-response curve (A) and time course (B) of ipsilateral rotation following administration of (+)-amphetamine to rats with a unilateral 6-OHDA lesion.</td>
<td>75</td>
</tr>
<tr>
<td>2.7 Diagram showing the positions in the left and right striatum at which GFAP-positive cells were counted.</td>
<td>82</td>
</tr>
<tr>
<td>2.8 Sample standard curves for (A) L-DOPA, (B) 3-OMD, (C) DA, (D) DOPAC, and (E) HVA, used to calibrate the HPLC system.</td>
<td>86</td>
</tr>
<tr>
<td>2.9 Recovery of L-DOPA, DA, DOPAC, 3-OMD and HVA from supernatants of homogenates prepared from (A) cerebellum and (B) plasma of normal rats.</td>
<td>89</td>
</tr>
<tr>
<td>2.10 Sample chromatograms from aliquots (50μl) of standard solutions (A) and supernatants from homogenates of intact (B) and 6-OHDA lesioned (C) rat striatum (B and C).</td>
<td>91</td>
</tr>
</tbody>
</table>
Figures Contd. PAGE

2.11. Saturation binding of $^3$H-spiperone to striatal slices (A), and Scatchard transformation (B) of the specific saturation binding data for $^3$H-spiperone. 95

2.12. Time course of association of $^3$H-spiperone (specific binding) to rat striatal sections (20μm) incubated in buffer containing 1.0 nM $^3$H-spiperone for times ranging from 5-90 minutes. 97

2.13. Time course of dissociation of $^3$H-spiperone (specific binding) from striatal slices. 98

2.14. Saturation binding of $^3$H-SCH 23390 to rat striatal slices (A), and Scatchard transformation (B) of the specific binding data for $^3$H-SCH 23390. 100

2.15. Time course of association of $^3$H-SCH 23390 (specific binding) to rat striatal sections. 102

2.16. Time course of dissociation of $^3$H-SCH 23390 (specific binding) from rat striatal sections. 103

2.17. Standard curve for autoradiographic computerised densitometry, using Amersham tritium standards, and analysed using the IBAS Kontron system. 108

2.18. Diagram demonstrating the sub-division of striatum into quadrants for densitometric analysis of autoradiograms. 109

2.19. Diagram showing the destination of animals used for the experiments described in this thesis. 112

2.20. Time course showing the points at which different procedures were carried out, and at which animals were allocated to further studies. 113

3.1. The mean fluid intake of 6-OHDA lesioned rats before and after a sham- (groups A and B) or foetal-graft (groups C and D), with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment for 5 weeks. 121

3.2. The mean body weight of 6-OHDA lesioned rats before and after a sham graft (groups A and B) or foetal graft surgery (groups C and D), with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment for 5 weeks. 123
3.3A. The mean (+)-amphetamine (5mg/kg ip)-induced rotational rates over a 1 hour period in 6-OHDA lesioned rats before and after sham graft (groups A and B) or foetal graft (groups C and D) with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment. 126

3.3B. The time course of rotational response to (+)-amphetamine (5mg/kg ip) in 6-OHDA lesioned rats before and 6 weeks after a sham graft (groups A and B) or a foetal graft (groups C and D), with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment. 126

3.4. The mean rotation rates over 30 minutes immediately following administration of apomorphine (0.5mg/kg sc) in 6-OHDA-lesioned rats before and after a sham graft (groups A and B) or foetal graft (groups C and D), with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment. 129

4.1. Photomicrographs of TH-immunostained cells located in a foetal ventral mesencephalic implant in an animal treated with L-DOPA and carbidopa. 149

4.2. High power view of a fine calibre TH-positive fibre taken from a graft of an animal treated with L-DOPA and carbidopa (different rat from that in Fig. 4.1). The TH-positive cells were located at the ventrolateral edge of a graft and the fibre shows multiple varicosities along its length. 151

4.3. High magnification of a single TH-positive cell found in a graft from a 6-OHDA-lesioned animal treated with L-DOPA and carbidopa. Several coarse branching processes, probably dendrites, can be seen. 152

4.4. Colour photographs of autoradiographs of specific $^3$H-mazindol binding in striatal sections of animals with a unilateral 6-OHDA lesion followed by sham graft (A) or foetal graft (B). 154

5.1. Pseudocolour transformation of specific binding of 0.4nM $^3$H-spirperone to striatal sections in images
Figures Contd.  

derived from autoradiographs of coronal forebrain sections taken through Level 2. 173

5.2. The percentage difference in specific $^3$H-spike-rone binding density between the left (6-OHDA lesioned and sham- or foetal-grafted) and right (intact) striatum is shown for each of the 3 levels analysed, for all groups of animals. 179

5.3. Pseudocolour transformation of specific binding of 0.2nM $^3$H-SCH 233990 to striatal sections in images derived from autoradiographs of coronal forebrain sections taken through Level 2. 184

5.4. The percentage difference in specific $^3$H-SCH 23390 binding density between the left (6-OHDA lesioned and sham- or foetal-grafted) and right (intact) striatum is shown for each of the 3 levels analysed, for all groups of animals. 190

6.1. Time course of surgical procedures, L-DOPA treatment and behavioural testing. 206

6.2. Mean fluid intake before, during and after L-DOPA treatment period. 209

6.3. Mean body weights of all groups of animals over L-DOPA treatment period. 210

6.4A. Mean (+)-amphetamine induced rotation at various time points over the study period is shown in all groups. 213

6.4B. Time course of (+)-amphetamine-induced rotation over 1 hour test periods at various times over the experimental period. 214

6.5. TH-immunohistochemistry of a foetal ventral mesencephalic graft in a rat treated with L-DOPA and carbidopa. 221

6.6. GFAP-immunohistochemistry of the right and left striata of a rat receiving a foetal graft (graft not shown). 225

6.7. GFAP immunohistochemical staining of the striatum containing a foetal VM graft without (A-C) or with L-DOPA treatment (D). 227
ABBREVIATIONS

ACh/E  acetycholine/esterase
AIS  apomorphine-induced stereotypy
BBB  blood brain barrier
CA  catecholamines
CNS  central nervous system
CPM  counts per minute
DOPAC  3,4-dihydroxyphenylacetic acid
DA  dopamine/dopaminergic
DHBA  dihydroxybenzylamine
DMI  desmethylimipramine
DPM  degradations per minute
EDTA  ethylenediaminetetra-acetic acid
GABA  gamma-aminobutyric acid
GFAP  glial fibrillary acidic protein
glu  glutamate
HPLC  high performance liquid chromatography
HVA  homovanillic acid
5HT  5-hydroxytryptamine
6-OHDA  6-hydroxydopamine
IHC  immunohistochemistry
LGP  lateral globus pallidus
L-DOPA  L-3,4-dihydroxyphenylalanine
MHC  major histocompatability locus
MFB  medial forebrain bundle
MGP  medial globus pallidus
MPTP  1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NGF  nerve growth factor
NGS  normal goat serum
PBS  phosphate buffered saline
PD  Parkinson’s disease
SN  substantia nigra
SNC  substantia nigra compacta
SNR  substantia nigra reticulata
STN  subthalamic nucleus
TH  tyrosine hydroxylase
VM  ventral mesencephalic/mesencephalon
VTA  ventral tegmental area


SB Blunt, P Jenner, CD Marsden, Motor function, graft survival and gliosis in rats with 6-OHDA lesions and foetal ventral mesencephalic grafts chronically treated with L-DOPA and carbidopa. Experimental Brain Research (In Press).

SB Blunt, P Jenner, CD Marsden, Autoradiographic study of striatal D-1 and D-2 dopamine receptors in 6-OHDA lesioned rats receiving foetal ventral mesencephalic grafts and chronic treatment with L-DOPA and carbidopa. Submitted to Brain Research.
Chapter 1

GENERAL INTRODUCTION
1.1. PARKINSON’S DISEASE

1.1.1. Definition, Clinical Features and Diagnosis

"Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported: with a propensity to bend the trunk forward, and to pass from a walking to a running pace: the senses and intellect being uninjured '. This definition opens the book entitled 'An Essay On The Shaking Palsy' (1817), written by the apothecary and surgeon, James Parkinson. In the book, Parkinson goes on to describe all the features of the untreated disease in 6 graphic case reports. Today the disease aptly bears his name. Parkinson's disease is characterised by a disturbance of motor function with slowing of voluntary and emotional movements (bradykinesia), muscular rigidity and tremor. These features are collectively referred to as 'parkinsonism'. A definitive diagnosis of Parkinson's disease can only be made at post mortem, with the identification of Lewy bodies in the dopaminergic neurones remaining in the substantia nigra (SN) (section 1.1.4). The most reliable clinical features for predicting Lewy-body positive parkinsonism ('Parkinson's disease') are a unilateral onset of symptoms, classic rest tremor (4-6 Hz), and a pronounced response to L-3,4-dihydroxyphenylalanine (L-DOPA) (Marsden 1990). Parkinsonism unresponsive to L-DOPA, and occurring in association with signs of more widespread central nervous system (CNS) pathology, comes under the category of 'parkinsonism-plus syndromes.' Examples of these include multiple system atrophies (striato-nigral degeneration, olivoponto-cerebellar atrophy and Shy Drager syndrome), progressive supranuclear palsy and corticobasal degeneration (Jancovic 1989). About 10-15% of cases of parkinsonism fall into these categories.

1.1.2. Epidemiology and Aetiology

Parkinson's disease is the most common disease affecting
the basal ganglia. Its prevalence is approximately 100/100,000 population and increases with age until the age of 75 (Hoehn and Yahr 1967). The incidence is approximately 20 cases per 100,000 individuals per year and peaks in the 5th-6th decades. The cause of Parkinson's disease is unknown, but epidemiological studies may give some clue.

Parkinson's disease occurs equally in blacks and whites living in rural America, but is less common in blacks living in rural West Africa (Schoenberg 1987). These findings suggest that racial factors may be unimportant. The role of genetic factors is uncertain, but Parkinson's disease occurs more frequently in some families (Roy et al 1983; Maraganore et al 1991). However, studies of monozygotic twins suggest that a pure heredity role is unlikely (Ward et al 1983, Marsden 1987). Attention to a possible environmental toxin as a cause of Parkinson's disease increased with the discovery that the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which contaminated drugs used by drug abusers in California, caused a parkinsonian syndrome (Davies et al 1979; Burns et al 1983). The active metabolite MPP⁺ is chemically related to the pesticide Paraquat. Lewin (1985) reported an association between pesticide use and the incidence of Parkinson's disease. A well-water-borne agent has been implicated (Rajput et al 1987) but this association was not confirmed in a large case-control study (Semchuk et al 1991). Interestingly, an inverse relationship between smoking and mortality from Parkinson's disease has been reported (Schoenberg 1987).

The possibility that an environmental toxin might be the cause of Parkinson's disease becomes more plausible if coupled with the concept that some individuals may be more susceptible to certain compounds, through an inherent metabolic defect. There is increasing evidence that patients with Parkinson's disease have impaired
metabolism of certain foreign compounds. In 1985, Barbeau et al suggested that the 4-hydroxylation of debrisoquine by a cytochrome P450 isoenzyme was decreased in patients with Parkinson's disease, especially of early-onset. Subsequent studies, however, failed to show any abnormality of debrisoquine metabolism (Steventon et al 1989; Benitez et al 1990).

Some patients with Parkinson's disease have abnormalities of sulphur metabolism. The activity of hepatic cysteine dioxygenase is reduced in Parkinson's disease. This enzyme catalyses sulphoxidation of S-carbocysteine, and its deficiency could lead to increased cysteine levels and a reduced sulphate pool for detoxification reactions (Steventon et al 1989). Increased cysteine levels might chelate iron through the thiol group, so producing increased levels of iron in brains of patients with Parkinson's disease (Dexter et al 1989). As described below, excess iron may stimulate formation of toxic species. A reduced activity of a second enzyme involved in sulphur metabolism, thiolmethyl-transferase, has also been demonstrated, and this could increase susceptibility to thiol toxicity (Waring et al 1989). However, similar abnormalities in sulphur metabolism have also been found in rheumatoid arthritis and ulcerative colitis (Waring et al 1989).

Iron can stimulate the formation of various toxic substances including \( \text{H}_2\text{O}_2 \), \( \text{O}^2^- \) and \( \text{OH}^- \) (Halliwell 1989). Increases in brain iron levels through abnormal metabolism or decreased sequestration into ferritin may stimulate cell death. Iron has an uneven distribution in the brain, with high concentrations in the globus pallidus, caudate-putamen and SN (Spatz, 1922). There have been reports of increased iron levels in the SN in Parkinson's disease (Dexter et al 1988; Riederer et al 1988), and the \( \text{Fe}^{2+}/\text{Fe}^{3+} \) ratio is decreased, suggesting an alteration in the oxidative state of the brain (Sofic

22
et al 1988). However, some studies have failed to show any increase in iron levels (Uitti et al 1989). Decreased levels of ferritin in the SN disease have also been observed in Parkinson's disease (Dexter et al 1991), so the increased iron levels could be present in a free and reactive form. However, others have shown increased ferritin levels in the SN (Riederer et al 1989). So, Parkinson's disease may be associated with abnormal iron metabolism which could contribute to its pathology.

Another possible endogenous cause of Parkinson's disease could be toxic substances produced by dopamine (DA) metabolism. As described in detail in section 1.3.4.2, normal metabolism of DA produces hydrogen peroxide ($\text{H}_2\text{O}_2$), whilst auto-oxidation can result in production of the toxin 6-hydroxydopamine (6-OHDA), semiquinones, $\text{H}_2\text{O}_2$ and superoxide radical ($\text{O}_2^-$), which in turn may lead to formation of the highly toxic hydroxy radical ($\text{OH}^-$). The role of these substances in neuronal death in Parkinson's disease is not clear. Since not all areas of the brain which degenerate in Parkinson's disease contain catecholamines (section 1.1.5), it is unlikely that the auto-oxidation pathways are a major cause of cell death.

1.1.3. Anatomy of Parkinson's Disease
Basal ganglia anatomy and transmitter systems have been reviewed by Albin et al (1989): the basal ganglia are a group of interconnected subcortical nuclei which also connect to cortical structures. The primary afferent structure is the striatum which is a single structure in the rat. In most other animals the striatum consists of two parts - the medial caudate and the lateral putamen, separated by fibres of the internal capsule. The main output structures of the striatum are the medial globus pallidus (MGP) or 'interna' and the substantia nigra reticulata (SNr). These nuclei contain similar neurone types but are separated by fibres of the cerebral peduncles. In most mammals the homologue of the MGP is
the entopeduncular nucleus (EP). The major pathways to and from the various nuclei and the transmitters involved are shown diagramatically in Figure 1.1.

Dopaminergic activity in the substantia nigra compacta (SNc) is regulated primarily by the striatonigral feedback loop, and self-regulation through autoreceptors (located on dendrites in the SNc and nerve terminals in the striatum). SNc dopaminergic nerve terminals synapse on striatal projection neurones directly, but also interact with corticostriatal nerve terminals and striatal interneurones. In Parkinson's disease, DA neurones in the SN, which project to both the striosomal (inhibitory) and matrix (excitatory) projection neurones in the striatum, degenerate. This loss of input results in overactivity of striatal projections to the lateral globus pallidus (LGP) (their DA input being inhibitory) whilst projections to the MGP, SNr and SNc become less active (their DA input being excitatory). The reduction of striatal GABAergic activity to the MGP and SNr results in their increased output to the ventrolateral thalamus with inhibition of the thalamocortical projection. Terminal arborisations of individual DA nerve terminals in the striatum are extensive (Bjorklund and Lindvall 1986), so partial lesions can have widespread effects on striatal activity. However, remaining neurones compensate through hyperactivity and symptoms do not appear until considerable loss of striatal dopamine has developed (Hornykiewicz and Kish 1986).

1.1.4. Pathology of Parkinson's disease
Parkinson's disease has a characteristic pathology (Forno 1982, 1986). Pigmented DA neurones in the SNc degenerate, and characteristic intraneuronal perikaryal eosinophilic inclusions, known as Lewy bodies (Lewy 1913), are formed. Dopamine cell loss is not uniform within the SN, being most marked in the caudal and ventrolateral portions
Figure 1.1. Basal ganglia connections and transmitter systems (Albin et al. 1989)
The striatum is composed mainly of projection neurones and small interneurones (not shown). The projection neurones have highly collateralised axons with direct projections to the medial globus pallidus (MGP), SNr and SNC. The striatum receives a rich innervation from the SNC. Indirect projections to the MGP and SNr also occur via direct striatal projections to the lateral globus pallidus (LGP). The LGP sends a large projection to the subthalamic nucleus (STN) which in turn projects back to the MGP, SNr and LGP. The striatum also receives afferents from the isocortex and from serotonin neurones in the dorsal raphe nuclei (not shown). The main output structures are the MGP and SNr which send afferents to the ventrolateral thalamic nucleus. Thalamic projections go to the prefrontal cortex and the supplementary motor cortex. The striatum is divided into compartments based on the intensity of histochemical staining for AChE (acetylcholine esterase) in primates, and on the distribution of opiate (μ) receptors in rodents (Graybiel and Ragsdale 1978, Herkenham and Pert 1981). Areas of intense μ receptor density and AChE staining are called striosomes, and the areas of lower intensity are called the matrix. These compartments have different afferent and efferent connections and may contain different transmitters. The striosomes receive cortical afferents from prefrontal and limbic cortex, while the matrix receives cortical afferents from the primary motor and somatosensory cortex as well as frontal, parietal and occipital cortex. Similarly, discrete subgroups of dopamine neurones in the SNC (not shown) project to striosomes or matrix (Gerfen et al. 1987a,b; Jimenez-Castellanos and Graybiel 1987).

The major transmitter of striatal, pallidal and SNr projection neurones is γ-aminobutyric acid (GABA), and its functions are probably inhibitory. Interneurones in the striatum contain Ach, somatostatin and neuropeptide γ. Cholinergic interneurones are excitatory to GABA projection neurones. The striatal afferents from the cortex are mainly glutaminergic (glu) and excitatory, whilst those from the SNC are dopaminergic (DA) and inhibitory. Other putative neurotransmitters include enkephalin (enk) and substance P.
of the SN (Bernheimer et al, 1973). Neuromelanin is released from the dying cells and removed by macrophages. Pathological processes are also found in other pigmented nuclei such as the ventral tegmental area (VTA), locus coeruleus and dorsal vagus motor nucleus. In addition, neuronal loss occurs in non-pigmented nuclei in the substantia innominata, hypothalamus, dorsal raphe nucleus, sympathetic ganglia and spinal cord. Lewy bodies are also found in the cerebral cortex (Ikeda et al 1978).

Lewy bodies are not pathognomonic for Parkinson's disease. They can be found 'incidentally' in the brains of apparently normal individuals over 60 who have no signs of Parkinson's disease in life (Forno 1986; Gibb 1989). Such cases may represent presymptomatic disease. Lewy bodies have also been found in other basal ganglia disorders: Hallervorden Spatz disease, neuroaxonal dystrophy, ataxia telangiectasia, strionigral degeneration and progressive supranuclear palsy (Gibb and Lees 1988). Filamentous aggregates similar to Lewy bodies have been induced experimentally with MPTP (Forno, 1986; Gibb and Lees 1988). The pattern of DA neuronal loss in Parkinson's disease differs from that found in normal ageing (Fearnley and Lees, in Press); and from that found in post-encephalitic parkinsonism where there is diffuse degeneration and gliosis of the SN and locus coeruleus, and an absence of Lewy bodies (Jellinger 1987; McGeer et al 1988).

1.1.5. Biochemistry of Parkinson's Disease

In 1960, Ehringer and Hornykiewicz demonstrated reduced DA concentrations in the striatum of parkinsonian patients. Loss of DA neurones in the SN is the cause of the decreased DA content of the striatum. Most cell loss occurs in the caudal and ventrolateral SN, which project preferentially to the putamen (Bernheimer et al, 1973). This may explain why reduction in DA concentration is
greater in the putamen than in the caudate (Kish et al 1986). There is a rostro-caudal gradient of DA loss in the putamen (greater loss caudally) whilst the reduction in the caudate runs in the opposite direction (Kish et al 1986, 1988). The dorsal regions of both nuclei are more severely depleted. DA metabolites (3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) are also reduced in the SN and striatum although the HVA/DA ratio is increased, suggesting a compensatory increase in DA turnover of the surviving neurones (Agid et al 1987).

Other DA systems are damaged, but to a much lesser extent than the nigrostriatal system (Agid et al 1987). Degeneration of DA neurones in the VTA projecting to limbic areas, neocortical areas and hypothalamus, globus pallidus and subthalamic nucleus is also found. The only DA neurones which appear to be spared are those projecting from the caudal hypothalamus to the lumbar spinal cord (Scatton et al 1986). Peripheral catecholamine (CA) neurones in the adrenal medulla may also be involved in the disease (Stoddard et al 1989).

The long ascending pathways of the noradrenergic, serotonergic and cholinergic systems, originating respectively in the locus coeruleus, dorsal raphe nucleus and nucleus basalis of Meynert may be affected in Parkinson's disease (Jellinger et al 1987). Reductions in cholecystokinin-8-like and substance P-like immunoreactivity have also been found in the SN in Parkinson's disease. Met-enkephalin-like immunostaining (whose distribution normally parallels closely that of dopamine) is also reduced in the SN and VTA (Agid et al 1987), although the mosaic pattern of Met-enkephalin-like immunoreactivity in the striatum is preserved in Parkinson's disease (Goto et al 1990).
1.1.6. Dopamine receptor changes in Parkinson's disease

The investigation of DA receptors in Parkinson's disease is complicated by differences in disease state and history of drug treatment at the time of study. The literature addressing the status of DA D-1 and D-2 receptors in Parkinson's disease reports conflicting findings. Post mortem studies using in vitro homogenate binding have revealed increases in D-2 density in the putamen of patients not treated with L-DOPA (Rinne 1982, Lee et al 1978, Guttman and Seeman 1985, Seeman et al 1987) as well as decreases which may be related to the stage of disease (Rinne 1982). L-DOPA treatment has been associated with normal or reduced D-2 densities (Rinne 1982, Pimoule et al 1985, Guttman and Seeman 1985, Cash et al 1987) or increased densities in the putamen (Bokobza et al 1984). Autoradiographic studies have shown no difference between patients with Parkinson's disease and normal controls for either D-1 or D-2 receptors, and L-DOPA treatment was also without effect (Kito et al 1986, Cortes et al 1989). Positron emission tomography has shown no difference in D-2 density between patients and normal age-matched controls (Wagner 1986, Lindvall et al 1987) or increases which are reduced in patients treated with L-DOPA (Leenders 1988). Others found increased densities in patients treated with L-DOPA (Wijnand et al 1987; Rutger et al 1987).

1.1.7. Drug Treatment of Parkinson's Disease

1.1.7.1. Drugs commonly used

The most effective symptomatic treatment of Parkinson's disease consists of the drug L-DOPA together with a peripheral dopa decarboxylase inhibitor (section 1.1.7.2.). Prior to the L-DOPA era, the principal drugs used to treat Parkinson's disease comprised amantadine and anticholinergics. These drugs are still used in the early treatment of mild parkinsonism (Marsden and Fahn 1987). Other drugs now used, or under study, include
direct DA agonists such as bromocriptine, lisuride, pergolide and apomorphine. Deprenyl, a monoamine oxidase type B (MAO-B) inhibitor, was introduced initially as symptomatic treatment for Parkinson's disease. MAO-B catabolises DA in the brain, so the initial rationale was that inhibition of this enzyme would prolong the action of administered L-DOPA, so improving fluctuations in response. Some patients with the 'wearing off' effect are undoubtedly improved by this drug (Marsden and Fahn 1987). One of the many by-products, however, of the discovery of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was the observation that deprenyl could prevent experimentally-induced MPTP toxicity to DA neurones (Cohen et al 1984). MAO-B is involved in the metabolism of MPTP to MPP⁺, so inhibition of this enzyme prevents formation of the toxic species. If Parkinson's disease is caused by an agent resembling MPP⁺, deprenyl could have a similarly beneficial effect by preventing or slowing further cellular damage. Also, MAO-B inhibitors may reduce OH⁻ production from DA metabolism. Indeed, deprenyl may slow clinical deterioration in Parkinson's disease (Birkmayer and Riederer, 1984; Tetrud and Langston, 1989; Shoulson et al, 1989).

1.1.7.2. Treatment with L-DOPA

The DA precursor D,L-3,4-dihydroxyphenylalanine (D,L-DOPA) was introduced for the treatment of Parkinson's disease in 1967 and produced sustained improvement in many patients (Cotzias et al 1967). Most marked was the effect on akinesia and rigidity. However, side effects of vomiting and postural hypotension were occasionally so severe that the dose could not be increased sufficiently to produce a therapeutic effect. A more worrying problem was granulocytopenia. Because of these side effects, the therapeutic and toxic effects of the L isomer, L-DOPA, were investigated (Cotzias et al 1969). The short term benefits of L-DOPA included improvements in akinesia,
rigidity and tremor. In addition, associated movements, shuffling, dysphagia, aphonia, articulation, diapheresis, lacrimation, salivation, seborrhoea, ankle oedema, handwriting, posture, festination and facial expression were improved.

By comparison with the D,L compound, L-DOPA could be used at half the dose, caused few cases of granulocytopenia, and led to improvement in a higher proportion of patients. However, side effects of L-DOPA included nausea and vomiting, cardiac arrhythmias, involuntary movements (myoclonus, chorea, facial grimacing) and labile hypertension. Most of these side effects (labile blood pressure, arrhythmias and nausea/vomiting) resulted from peripheral conversion of L-DOPA to DA by dopa decarboxylase, and these were reduced by administration of the peripheral dopa decarboxylase inhibitor α-methyl dopahydrazine (Cotzias et al 1969). This drug does not cross the blood brain barrier (BBB) and so inhibits only the peripheral conversion of dopa to DA. α-methyl dopahydrazine potentiates the degree to which a given dose of L-DOPA will elevate brain DA levels, whilst minimising peripheral side effects of DA. Combined treatment of patients with L-DOPA and carbidopa (α-methyldopahydrazine) (introduced in the early 1970's) increased the rate at which optimum dosage and benefit were obtained, and allowed a 4-fold reduction in dose of L-DOPA (Marsden and Parkes 1977).

L-DOPA is effective in more than two-thirds of patients in the short term (Marsden and Parkes 1977), and it restores life expectancy to normal (Shaw et al 1980). However, studies of prolonged treatment with L-DOPA show that after 2-15 years of treatment, beneficial response to the drug wanes as the underlying disease progresses (Hunter et al 1973; Marsden and Parkes 1977). After 3 years of treatment, only 40% of patients maintain their initial improvement, and after a static period of 2
years, most patients continue to deteriorate (Hunter et al 1973). The earliest sign of decreased efficacy is the 'wearing off' effect, where the duration of benefit that usually follows a given dose of L-DOPA decreases.

Side effects of chronic L-DOPA treatment also appear. Although some fluctuations in motor performance (in the form of dystonia or freezing attacks) can occur in untreated Parkinson's disease (Marsden 1980a), others occur only in patients treated with L-DOPA. After 2 years of treatment, most patients develop involuntary movements and fluctuations in motor performance (Marsden and Parkes 1977). Shaw and colleagues (1980) showed that after 6 years of treatment with L-DOPA, peak-dose dyskinesias occurred in 80% of patients, early morning and end-of-dose dystonia in 20%, and biphasic dyskinesia in 3%. They also found a high incidence of mental symptoms such as psychosis, dementia, and sleep abnormalities. 'Wearing-off' occurred in 65% and the 'on-off' phenomenon (in which the patient changes suddenly from a period of immobility (the 'off' phase) to one of normal or excessive mobility (the 'on' phase) in 10%.

A variety of dykinesias have been associated with prolonged L-DOPA treatment (Marsden 1980a), including choreiform movements of the orofacial muscles and, less often, dystonia. Dyskinesias may occur either at the height of the beneficial effect of a dose (ie 'peak-dose dyskinesia') or prior to its onset and/or as the beneficial effect begins to wear off ('biphasic dyskinesia'). L-DOPA-induced dyskinesias do not occur in normal individuals, and they may reflect altered postsynaptic DA receptor physiology in parkinsonian patients (Lee et al 1978). Similarly, normal monkeys treated chronically with L-DOPA do not develop dyskinesias, whereas those treated previously with MPTP do (Clarke et al 1987; Falardeau et al 1988). Thus, for the appearance of L-DOPA-induced dyskinesias, chronic
dopamine-denervation of the striatum appears to be required. However, chronic L-DOPA treatment itself may play a part in the development of dyskinesias, as their prevalence correlates both with the total amount of drug ingested and the duration of treatment (Quinn et al 1986a; Mouradian et al 1988). However, this correlation was not confirmed by a recent study (Cedarbaum et al 1991). Dystonic phenomena related to chronic L-DOPA treatment include end-of-dose and early morning dystonia (Lees et al 1977), which may reflect drug-induced post-synaptic DA receptor supersensitivity (Hardie 1989).

Efforts have been made to counter the reducing efficacy of L-DOPA and the side effects associated with its long-term use. Attempts to combat the 'wearing off' effect include closer spacing of doses of L-DOPA, the addition of a direct DA agonist (such as bromocriptine), and the addition of deprenyl. On-off phenomena have been successfully treated with continuous intravenous infusions of L-DOPA or lisuride (Quinn et al 1984; Obeso et al 1983; Hardie et al 1984), and more recently with subcutaneous apomorphine injections (Stibe et al 1988). Repeated infusions with L-DOPA on consecutive days are also effective in the treatment of 'on-off' phenomena (Marion et al 1986). Peak-dose dyskinesias respond to reductions in dose of L-DOPA at the expense of some loss of therapeutic benefit (Marsden and Fahn 1987). Early introduction of direct DA agonists such as bromocriptine either as a first line treatment or in combination with L-DOPA may delay the appearance of dyskinesias (Rinne et al 1989).

A further potential problem related to treatment with L-DOPA is the concern that auto-oxidation of DA may produce metabolites which are toxic to CA cells (Cohen 1986). This is discussed in section 1.3.4.2.
1.1.8. Neural transplantation - A novel approach to treatment
Because of the problems associated with long-term L-DOPA treatment - not only in terms of side-effects, but also because of progression of the underlying disease, alternative treatments have been sought. One approach involves the transplantation of healthy DA cells derived from foetal ventral mesencephalon (VM) into the parkinsonian striatum, or into the adjacent lateral ventricle. Other possible sources of DA currently under investigation include adrenal medulla chromaffin cells (reviewed by W. Freed et al 1990), cells (such as fibroblasts) genetically modified to produce DA (Uchida et al 1990; Gage et al 1990), and synthetic polymers which result in a slow delivery of contained DA (Jaeger et al 1990). The experimental background to the transplantation of adrenal medulla and foetal ventral mesencephalic (VM) cells, and their application to man are discussed in section 1.3. An animal model widely used for the experimental study of these grafts is outlined below.

1.2. ANIMAL MODELS OF PARKINSON'S DISEASE

Parkinson's disease is thought not to occur spontaneously in animals. Therefore, animal models of the disease have been developed by reproducing some of the major biochemical changes found in the human disease. However, the models differ in important ways from the human condition. First, as the cause of nigral cell damage in Parkinson's disease is unknown, the same process cannot be reproduced in animals. The pathological damage produced by MPTP in primates may be the closest model of the human disease (see below). Second, the time course over which cell damage occurs is usually acute and finite in the animal models, whereas in Parkinson's disease this may be progressive (Bernheimer et al 1973). Chronic administration of MPTP is an attempt to reproduce this in
the experimental situation. Third, the behavioural changes produced by nigrostriatal damage in rats are different from the clinical features of Parkinson's disease. Despite these differences, animal models are useful for monitoring changes associated with striatal DA depletion, as described below in relation to the rat model.

1.2.1. A Rat model of Parkinson's disease - Unilateral 6-OHDA lesion of the SN or nigrostriatal pathway

Early models of Parkinson's disease involved the use of drugs acting presynaptically by inhibiting synthesis (α-methyl-paratyrosine) or storage (reserpine or tetrabenazine) of CAs. Other drugs which act by antagonising post-synaptic DA receptors (such as phenothiazines or butyrophenones) have also been used (Marsden 1980b). Destruction of the DA cell bodies in the SN or the emerging fibres in the medial forebrain bundle (MFB) by a chemical or a surgical lesion produces a permanent depletion of DA in the ipsilateral striatum. Unilateral depletion of striatal DA by a 6-hydroxydopamine (6-OHDA) lesion of the nigrostriatal pathway forms the basis of the rotating rodent model of hemiparkinsonism (Ungerstedt 1968). This model is used in this thesis and its characteristics will be outlined.

A 6-OHDA lesion of the MFB damages neurones from the ipsilateral SN and ventral tegmental area (VTA) (Ungerstedt 1971a). The axons gather together in the lateral hypothalamus, enter the internal capsule, fan out in the globus pallidus and terminate in the striatum. Fibres from the SN preferentially innervate the lateral striatum, whilst those from the VTA supply the medial parts and limbic areas (Bjorklund and Lindvall 1986). Dopaminergic innervation of the striatum is compartmentalised into striosomes and matrix (Gerfen et al 1987a, b). DA fibres from the cells in the VTA form the mesolimbico-cortical system and enter the MFB where
they lie medial to fibres of the nigrostriatal pathway. The mesolimbic fibres terminate in the nucleus accumbens and olfactory tubercle (Ungerstedt 1974). Some fibres from each SN cross the midline to innervate the contralateral striatum (Fass and Butcher 1981).

*Classical theory of the rotating rodent model:*

Unilateral injection of DA into one striatum produces spontaneous asymmetry in posture directed away from the side with the higher DA concentration (Ungerstedt et al 1969). Similarly, unilateral depletion of DA produces a postural deviation or spontaneous rotation of the animal away from the side with the higher DA concentration, i.e. towards the lesioned side, ('ipsilateral') Ungerstedt 1971b). Any manouevre which exaggerates the 6-OHDA-lesion-induced imbalance in DA levels between the 2 hemispheres exaggerates the spontaneous tendency for postural deviation and rotation towards the lesioned side. Thus, (+)-amphetamine, which acts presynaptically to release DA from the intact striatum (Glowinski 1975; McMillen 1983), increases the asymmetry to the point of marked rotation towards the lesioned side (Ungerstedt and Arbuthnott 1970; Ungerstedt 1971b). The rate of rotational response to (+)-amphetamine (1-5mg/kg ip) depends on the extent of the DA imbalance; severe lesions produce high rates of ipsilateral rotation, whilst lesser lesions produce correspondingly lower rates (Ungerstedt 1971b). However, Schmidt et al (1982) have shown that ipsilateral rotation of >7/min following metamphetamine 5mg/kg ip indicates >99% DA depletion in the ipsilateral striatum.

When lesions of the nigrostriatal pathway are near complete, post-synaptic DA receptor supersensitivity occurs, and behavioural supersensitivity to directly acting DA agonists is observed. This is the basis of apomorphine-induced circling behaviour (Ungerstedt 1971c;
apomorphine acts preferentially on the denervated receptors of the 6-OHDA-lesioned striatum, rotation occurs away from the lesioned hemisphere (i.e. 'contralateral'). These tests are used as screens for 'complete' lesions of the nigrostriatal pathway. One theory which explains the unilateral 6-OHDA lesion rotation model is that DA depletion in the ipsilateral striatum results in postural deviation towards the lesioned side, whilst DA released in the intact mesolimbic DA pathways (importantly the nucleus accumbens) accounts for the locomotor response which turns the postural asymmetry into a circling behaviour (Pycock and Marsden 1978). This model of nigrostriatal degeneration is reproducible and robust and can be quantified by counting the rate of rotation following administration of apomorphine or (+)-amphetamine. Changes in rotation rates can be monitored and used to assess the success of various procedures aimed at restoring the DA imbalance between the right and left sides, for example with neural transplants.

1.2.2. The MPTP primate model of Parkinson's disease
Treatment of monkeys with MPTP has resulted in the most accurate model of Parkinson's disease both in terms of its clinical features and its pathology (Burns et al 1983, Jenner et al 1984). MPP⁺, produced by oxidation of MPTP by MAO-B (Chiba et al 1984), probably in glia (Javitch et al 1985a), is taken up selectively by DA neurones, where it causes cell death. The neurotoxicity of MPTP varies between species, with rat DA cells showing minimal susceptibility. However, infusion of the active metabolite, MPP⁺, directly into the SN of rats can induce severe DA cell loss (Sirinathsinghi et al 1990). MPTP-treated mice have also been used as a model of Parkinson's disease (Bohn et al 1987). A major disadvantage of the MPTP monkey model for interpreting the effects of grafts is that some spontaneous
behavioural recovery occurs in the first months after the MPTP lesion (Waters et al. 1987; Kurlan et al. 1991).

1.3. ALTERNATIVE TREATMENT FOR PARKINSON'S DISEASE: NEURAL AND ADRENAL MEDULLA TRANSPLANTATION

Transplantation of foetal VM tissue and adrenal medulla tissue into the human brain is well underway. The clinical enthusiasm for such foetal neural grafting is based on its marked success in rats and, to a lesser extent, non-human primate models. This success is demonstrated by graft-induced behavioural recovery of many lesion-induced deficits, DA cell survival in the grafts, and evidence of biochemical and electrophysiological activity of the grafts. Animals experiments using adrenal tissue are less encouraging, both in terms of CA cell survival and behavioural recovery, with little basis for the large number of transplants that have been performed in man.

Rationale for transplantation
The rationale for transplantation of foetal VM cells in the treatment of Parkinson's disease stems from the fact that the rostral and lateral regions of this area will form the pars compacta of the adult SN (Nobin and Bjorklund 1973; Specht et al. 1981; Freeman et al. 1989). Transplanted cells might, especially if they formed appropriate connections with host neurones, deliver DA in a more physiological manner than that provided by exogenously administered L-DOPA. A successful operation might, at best, reverse the disease process (producing a 'cure'), and at worst avoid or dampen L-DOPA-related problems. The rationale for using adrenal medulla tissue in the treatment of Parkinson's disease derives from the observation that rat, monkey and human adrenal chromaffin cells produce small amounts of DA, and can adopt a neuronal phenotype if exposed to nerve growth factor (NGF) (Notter et al. 1986, 1989; Hansen et al. 1988a).
Transplantation techniques

Many different approaches to transplantation have been adopted both in experimental models and clinically. Rat foetal VM tissue has been implanted as solid pieces into the lateral ventricle (Perlow et al 1979), and into a previously prepared cavity in the dorsal striatum (Bjorklund and Stenevi 1979); or as a cell suspension directly into the striatal parenchyma (Bjorklund et al 1980a); or as solid pieces into the parenchyma stereotaxically (Steece-Collier et al 1990). Clinically, foetal tissue has been implanted as a cell suspension (Lindvall et al 1988-1990; CR Freed et al 1990); as larger tissue pieces by open microsurgical implantation into the caudate (Madrazo et al 1988, 1990a,b), or stereotaxically from older foetuses directly into the parenchyma of the striatum (Hitchcock et al 1988, 1990).

In animals, adrenal medulla tissue has been implanted into the lateral ventricle or into the striatal parenchyma as solid pieces, and into the striatum as a cell suspension (section 1.3.1.). Most human adrenal implants have used an open microsurgical approach with implantation of adrenal tissue into the caudate (Table 1.1). A few have involved stereotaxic implantation of tissue into the striatum.

1.3.1. Adrenal medulla transplants in animals
1.3.1.1. Studies in 6-OHDA-lesioned rats and MPTP-treated mice
Most experimental experience with adrenal medulla transplants derives from studies in rats. Reduction of drug-induced rotation following implantation of adrenal medulla into the lateral ventricle of 6-OHDA-lesioned rats was reported (Freed et al 1981), with survival of small numbers of CA cells. Subsequent studies with adrenal medulla implants in 6-OHDA-lesioned rats reported little behavioural recovery and poor cell survival (Herrera Marschitz et al 1984; Stromberg et al 1985;
survival of adrenal medulla tissue implanted into the rat striatum was enhanced by infusion of NGF, and some cells adopted a neuronal phenotype, and resulted in reduction of apomorphine-induced rotation (Herrera-Marschitz et al 1984; Stromberg et al 1985). Subsequently, adrenal medulla implants in MPTP-treated mice, either alone (Bohn et al 1987) or as cografts with peripheral nerve (Date et al 1990), induced sprouting of host nigrostriatal DA fibres.

1.3.1.2. Studies in non-human primates
Solid tissue or cell suspension grafts of adrenal medulla placed into the striatum of normal or MPTP-treated monkeys survived poorly (Morihisa et al 1984; Fiandaca et al 1988; Hansen et al 1988b). However, the adjacent striatum may exhibit robust sprouting of tyrosine-hydroxylase (TH)-positive fibres, a phenomenon also observed in sham-grafted animals (Fiandaca et al 1988). Co-grafts of adrenal medulla and sural nerves (whose Schwann cells produce NGF) implanted into the lesioned striatum of monkeys with unilateral MPTP lesions enhanced survival of adrenal chromaffin cells (Hansen et al 1990; Watts et al 1990), and induced sprouting of TH-positive fibres in the surrounding striatum (Watts et al 1990). These animals also demonstrated recovery in drug-induced rotation and skilled paw use (Watts et al 1990), although cell survival in the grafts was very small, and the relevance of that survival to behavioural recovery is unknown. Indeed, behavioural recovery was not observed in animals with surviving CA cells in the grafts but no sprouting of host TH-positive fibres in the striatum (Bankiewicz et al 1990a,b). This suggests that it may be the host sprouting which accounts for behavioural recovery. There is little evidence from these studies that adrenal medulla grafts result in behavioural recovery by secretion of DA (this being the original rationale for undertaking such studies).
1.3.2. Adrenal medulla transplants in man

Despite the poor experimental background, a large number of adrenal medulla auto-transplants have been performed in man (Table 1.1). There is also evidence that the adrenal medulla in some patients with Parkinson's disease contains reduced CA levels (Riederer et al 1977; Stoddard et al 1989). Most studies have shown minimal and shortlived motor improvement (usually less than 90 days) in patients receiving adrenal medulla grafts, and significant morbidity is associated with the abdominal surgery for adrenalectomy, and with the open microsurgical procedure for implantation into the brain (Goetz et al 1989). The reported success of Madrazo et al (1987a,b, 1990b), using intra-caudate grafts, has not been replicated. Most post mortem studies of patients receiving intra-striatal adrenal medulla implants have revealed no surviving CA cells (Peterson et al 1989; Hurtig et al 1989; Hirsch et al 1990). However, as in animal studies, adrenal medulla implants in man may stimulate recovery of host nigrostriatal DA fibres (Hirsch et al 1990; Kordover et al 1991).

Two recent reports suggest that adrenal medulla implants may produce longer lasting benefits than previously demonstrated. Olson et al (1991) described a patient with long-standing Parkinson's disease who received a stereotaxic adrenal medulla autograft into the putamen, supported by NGF infusion for 23 days post-operatively. As in earlier reports (Backlund et al 1985; Lindvall et al 1987), this patient demonstrated initial reduction in rigidity and hypokinesia over the first month, which declined over the second post-operative month. However, the patient went on to show a slower phase of improvement in motor function over the following 11 months. This protracted improvement was attributed to administration of NGF. However, Kordower et al (1991) also reported prolonged improvement (predominantly an increase in
Table 1.1. Adrenal medulla transplants in man.

<table>
<thead>
<tr>
<th>STUDY</th>
<th>METHOD &amp; SITE</th>
<th>NUMBER</th>
<th>OUTCOME</th>
<th>ASSESSMENT</th>
<th>MORBIDITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Backlund et al (1985)</td>
<td>stereotaxic intra-caudate implants of tissue pieces</td>
<td>2</td>
<td>mild improvement</td>
<td>self-scoring</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>in 1 patient for 4 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Madrazo et al (1987a)</td>
<td>open microsurgical intra-caudate implants</td>
<td>2</td>
<td>long-term improvement</td>
<td>self-scoring</td>
<td>-</td>
</tr>
<tr>
<td>Madrazo et al (1987b)</td>
<td></td>
<td>10</td>
<td>&quot;</td>
<td>&quot;</td>
<td>?</td>
</tr>
<tr>
<td>Penn et al (1988)</td>
<td></td>
<td>5</td>
<td>improvement over 20 weeks</td>
<td>IRSS psychological</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>respiratory</td>
<td></td>
</tr>
<tr>
<td>Ostrosky-Solis et al (1988)</td>
<td></td>
<td>7</td>
<td>immediate motor &amp; neuropsychol. improvement*</td>
<td>local rating</td>
<td>-</td>
</tr>
<tr>
<td>Peterson et al (1989)</td>
<td></td>
<td>1</td>
<td>short-lived motor improvement</td>
<td>self-scoring</td>
<td>death by 4 months, no CA cells at PM</td>
</tr>
<tr>
<td>Olanow et al (1990)</td>
<td></td>
<td>18</td>
<td>increased 'on' time and improved 'off' performance</td>
<td>IRSS perioperative performance</td>
<td></td>
</tr>
</tbody>
</table>

IRRS: internationally recognised scoring system
PM: post mortem

The Table summarises the reports of adrenal medulla transplants performed for the treatment of parkinsonism in man. Some of the studies have shown short-lived improvement in some motor symptoms, and one study has shown an apparent improvement in cognitive performance*. This change may have been related to alteration in L-DOPA treatment regime after surgery. There has been some morbidity in the immediate postoperative period, and few reports of lasting symptomatic improvement.
duration of 'on' and a decrease in disability during 'off' phases), persisting for 12-18 months after the implant, in a patient who received an intra-caudate adrenal medulla implant, but without NGF. However, the beneficial effect was not permanent, and the pre-operative level of motor function was once again attained at 30 months. Post mortem examination of the graft site at 30 months revealed surviving TH-positive cells (the first report of this), with sprouting of host TH-positive fibres in the adjacent striatum. So, adrenal medulla grafts may have a useful role in the transplantation treatment of Parkinson's disease, but the mechanism by which clinical improvement may occur is far from clear.

1.3.3. Neural transplantation in experimental animals

1.3.3.1. History
Studies of the transplantation of brain tissue into the central nervous system (CNS) date back to the turn of the century (Bjorklund and Stenevi 1985 for review). This early work lay almost forgotten until the 1970s when there was rapid development in the transplantation of foetal brain tissue into the brain or anterior chamber of the eye of laboratory animals, by several different laboratories (Das and Altman 1971; Bjorklund and Stenevi 1971; Olson and Seiger 1972).

1.3.3.2. Studies of rat foetal ventral mesencephalic (VM) grafts in 6-OHDA lesioned rats
The relevance of neural transplantation to the treatment of neurodegenerative diseases was brought to widespread attention by the pioneering studies of Bjorklund and Stenevi (1979) and Perlow et al (1979). Using the unilateral 6-OHDA lesioned rat model of Parkinson's disease, Perlow et al injected pieces of rat foetal VM into the lateral ventricle adjacent to the 6-OHDA-lesioned striatum. These grafts resulted in a reduction of apomorphine-induced contralateral rotation and large
numbers of surviving CA cells seen on histochemistry. Bjorklund and Stenevi (1979) transplanted foetal VM tissue from rat embryos of 16-19 days gestation into a cavity in the cortex over the 6-OHDA lesioned striatum. These grafts resulted in marked reduction of (+)-amphetamine-induced ipsilateral rotation with evidence of surviving implanted DA neurones and abundant fibre outgrowth into the denervated striatum. Using this technique, Bjorklund et al (1980a) showed that the reduction of amphetamine-induced ipsilateral rotation in the rat model brought about by the transplants correlated with the size of the DA fibre ingrowth from the grafts into the denervated striatum. Subsequent surgical removal of the graft reinstated the rotational behaviour.

The transplantation technique was later modified so that cell suspensions of foetal VM were injected stereotaxically into the 6-OHDA lesioned striatum (Bjorklund et al 1980a, 1983a). This allowed deeper sites to become accessible for graft placement. Using this technique, many of the parameters required for successful grafting and the amelioration of a variety of behavioural deficits were established. Some of the work which is of relevance to this thesis is summarised below:

(1) The distance over which foetal VM cell suspension grafts can extend their axons is limited. Thus, grafts placed into the 6-OHDA lesioned SN or at any point along the nigrostriatal trajectory do not extend their axons to reach the striatum from a distance (Bjorklund et al 1983b), unless 'bridges' of a suitable tissue such as peripheral nerve (Aguayo et al 1984) or embryonic striatal tissue (Dunnett et al 1989) are placed along the trajectory for the axons to grow along.

(2) Cell suspensions of foetal VM survive implantation into a variety of brain sites, but DA fibre outgrowth varies with the target area. Marked outgrowth occurs
only in areas normally receiving DA input (Bjorklund et al 1983a). Similarly, co-grafts of foetal striatal tissue and VM enhanced the DA fibre outgrowth from the VM tissue, with the foetal striatal tissue being preferentially innervated (Brundin et al 1986a; De Beaurepaire and Freed 1987; Yurek et al 1990). The presence of the 'normal' target tissue, either in the form of a co-graft, or as the implantation site itself, may provide a trophic environment for axonal extension.

(3) Topographical placement within the 6-OHDA lesioned striatum of a single graft determines which behavioural deficit resulting from the lesion will be improved (Dunnett et al 1981a, 1983). Thus, drug-induced rotation is most effectively reduced by a graft placed in the dorsal striatum, whilst sensorimotor deficits are relieved by a graft placed in the ventrolateral striatum. Multiple grafts can produce recovery in more than one behavioural test. Graft-derived recovery has been observed in several other lesion-induced deficits such as hypoactivity, contralateral sensory neglect and learning deficits (Dunnett et al 1981a, 1983, 1986, 1987; Nadaud et al 1984). However, some lesion-induced deficits which have escaped amelioration by foetal DA grafts include skilled independent paw use in reaching tasks (Dunnett et al 1987), and adipsia and aphagia which follow bilateral 6-OHDA lesions (Dunnett et al 1981b, 1983).

(4) A relationship exists between the extent of graft-derived reinnervation of the lesioned striatum and recovery of motor asymmetry. Graft-derived DA fibre reinnervation of only 1/8th of the head of the caudate putamen is sufficient to produce >90% reduction of amphetamine-induced ipsilateral rotation (Bjorklund and Stenevi 1979). A plateau then emerges where greater reinnervation produces no further behavioural effect. Likewise, amphetamine-induced ipsilateral rotation was significantly reduced (>50%) by foetal VM grafts (in the
dorsal striatum) containing 100-200 DA neurones, and could be detected 3 weeks after grafting (Brundin et al 1985a, 1988a). Grafts containing 300-400 DA neurones completely abolished amphetamine-induced rotation by 6 weeks after grafting. Survival of cells beyond this number produced a plateau effect with no further behavioural recovery. Dunnett et al (1988) showed that graft-derived behavioural recovery from a 6-OHDA lesion was specific to grafts rich in DA cells. Those authors also demonstrated that the extent of graft-derived reinnervation of the dorsal striatum correlated well with reduction in amphetamine-induced ipsilateral rotation, but less well with reduction of apomorphine-induced contralateral rotation.

(5) Foetal VM grafts form afferent (Bolam et al 1987; Doucet et al 1989a), and efferent (Freund et al 1985; Bolam et al 1987) synaptic connections with host striatal cells. Not all connections display normal morphology (Freund et al 1985; Gopinath et al 1991). Thus, whilst the graft-derived terminals form normal synapses with medium spiny neurons in the host striatum, those formed with magnocellular interneurones appear abnormal (Freund et al 1985).

(6) Foetal VM grafts are biochemically active. (+)-Amphetamine-induced ipsilateral rotation was abolished by graft-derived restoration of striatal DA levels to only 3% of normal (Schmidt et al 1982, 1983). Both spontaneous and drug-controlled release of DA from grafts occurs, as revealed by in vivo dialysis (Zetterstrom et al 1986; Strecker et al 1987) and in vitro studies of striatal slices (Carder et al 1989). Nishino et al (1990) showed with in vivo microdialysis that extracellular DA around cell suspension DA grafts recovered to 30-100% of control values. Functioning DA 'autoreceptors' (Roth 1984) may be present on grafted DA neurons, which may control local synthesis and release of DA (Strecker et al 1987).
vitro studies of superfused striatal slices show that foetal VM grafts restore inhibitory control over striatal cholinergic neurons (Herman et al 1988; Carder et al 1989). Solid grafts of foetal VM placed adjacent to the 6-OHDA-lesioned striatum produced a partial reversal of lesion-induced increase in striatal glutamic acid decarboxylase activity (Segovia et al 1989). These studies indicate that foetal grafts show some biochemical activity and control over striatal cells. Foetal VM grafts can reduce the increase in D-2 receptors that may develop after 6-OHDA or MPP⁺ lesions of the nigrostriatal pathway (Freed et al 1983; Sirinathsinghji et al 1990).

(7) Brundin et al (1985a, 1988a) investigated the limitations of rat foetal donor age and in vitro viability on subsequent graft survival and behavioural recovery. The best graft survival was obtained with VM tissue from foetuses of 13-15 days gestation. In vitro viability of foetal VM cells in suspension (using the vital stains acridine orange and ethidium bromide) was found to vary with foetal age. Suspensions from foetuses of 14-15 days gestation showed the greatest viability, and the slowest rate of decline with time. Rates of decline of viability varied with the brain region studied. For VM, viability remained close to 90% until 4 hours after trypsinisation, when it began to decline. In vitro viability was found to be a good predictor of in vivo graft survival and recovery of amphetamine-induced rotation for tissue of gestational age 14 or 15 days, but was less reliable for tissue of older gestational age. Tissue from foetuses older than 16 days gestation, or tissue that had been cultured for several days gave rise to very small grafts. However, modifications of technique, such as use of less mechanical dissociation and a wider bore injection cannula improved the ability of older tissue to survive.

(8) Allografts of CNS tissue into the brain survive
better than grafts to other parts of the body. This relatively immunoprivileged state of the brain has long been recognised (Barker and Billingham 1977). Possible reasons for this have been outlined by Mason et al (1985), and include: the absence of dendritic cells (important in presenting foreign antigen to lymph nodes); an incomplete lymphatic drainage system; and the fact that neuronal tissue does not normally express major histocompatibility (MHC) antigens (which allow recognition of 'self' and 'non-self'), although their expression can be induced under certain circumstances (Widner and Brundin 1988). Finally, an intact BBB may help to prevent effector cells gaining access to the grafted tissue from the blood.

(9) Whilst allografts can survive without rejection despite wide variability of MHC and non-MHC compatibility (Widner et al 1989), survival of xenografts is severely limited unless the recipient receives long-term immunosuppression (Brundin et al 1985b, 1988b, 1989a; Nakashima et al 1988). However, even within species, the immunological response to grafts may vary with transplantation technique. Solid grafts placed into a cavity overlying the striatum may evoke a different response to cell suspensions placed into the parenchyma, and to grafts placed into the lateral ventricle (Widner and Brundin 1988).

1.3.3.3. Studies of human foetal VM grafts in 6-OHDA-lesioned rats
Parameters for the successful transplantation of human foetal VM tissue have been studied in rats (Brundin et al 1985b, 1988b). Using dissociated cell suspensions, the best graft survival was obtained with foetal tissue of <9 weeks' gestation, and when immunosuppressive treatment was continued for prolonged periods. Cell suspension grafts of tissue from older foetuses of 15-19 weeks' gestation survived poorly. However, using solid tissue
grafts, others showed that older tissue (e.g. from foetuses aged 16 weeks gestation) survived implantation into the rat striatum (Stromberg et al 1989).

Cell suspension grafts from foetuses of 6.5-9 weeks' gestation implanted into the 6-OHDA lesioned striatum of immunosuppressed rats abolished amphetamine-induced ipsilateral rotation (Brundin et al 1986b, 1988b). The time course of this behavioural recovery differed from that found using rat foetal tissue, as reduction of amphetamine-induced rotation did not begin to appear until 11 weeks after grafting (compared with 4-6 weeks with rat tissue), and was fully developed at 19-20 weeks. Some reduction in apomorphine-induced contralateral rotation was observed 19 weeks after grafting with grafts of tissue from foetuses of <9 weeks gestation. However, Stromberg et al (1991) implanted human foetal tissue (7-12 weeks gestation) into the lateral ventricle adjacent to the 6-OHDA lesioned striatum and observed significant reduction in apomorphine-induced rotation at 2 months, which further decreased over time.

Morphological analysis of grafts of human VM tissue into rat brain reveals that the DA cells are larger than those found in rat foetal VM grafts, and that the distances covered by fibres from the grafts are greater. The time course of the development of synapses between human foetal tissue and the rat striatal cells correlates with the time course of reduction in amphetamine-induced rotation (Clarke et al 1988). Reciprocal synapses between rat host and human foetal DA grafts have been demonstrated (Stromberg et al 1988; Clarke et al 1988; Mahalik et al 1989). Human foetal VM grafts release DA (Brundin et al 1988b; Stromberg et al 1989), and are active electrophysiologically (Stromberg et al 1991).

1.3.3.4. Foetal ventral mesencephalic grafts in monkeys
There have been fewer studies of foetal primate VM grafts
in monkeys. Allografts of monkey foetal VM tissue implanted into the striatum of MPTP-treated adult monkeys, either as solid tissue pieces or as cell suspensions, resulted in some behavioural recovery with evidence of surviving TH-positive cells in the graft, in the absence of immunosuppression (Redmond et al 1986; Sladek et al 1986; Fine et al 1988). In addition, monkey foetal (Collier et al 1987) and human (Redmond et al 1988) foetal VM cells have survived grafting into the striatum of normal monkeys after a period of cryopreservation (Collier et al 1987). Monkey DA cell grafts implanted into the 6-OHDA-lesioned (Annett et al 1990), or MPTP-lesioned (Taylor et al 1990) striatum of monkeys produced recovery of spontaneous rotation, skilled paw use, and cognitive deficits, as well as improvement in parkinsonian symptoms (Taylor et al 1990). However, some lesion-induced deficits such as contralateral neglect (Annett et al 1990), ipsilateral hand preference (Annett et al 1990; Bankiewicz et al 1990), and apomorphine-induced rotation (Bankiewicz et al 1990) were not affected by the grafts. So, as in rat studies, foetal DA cell grafts in monkeys produce an incomplete profile of recovery of lesion-induced deficits.

1.3.4. Foetal ventral mesencephalic transplants in man.
Transplants of foetal neural tissue into the brains of patients with parkinsonism have been performed in Mexico, Birmingham, Sweden, and Denver and Poland (Table 1.2). Comparisons and interpretation of the purported changes are complicated by different methods of transplantation, different criteria for patient selection, and changes in drug regimes perioperatively in some cases.

1.3.4.1. Summary of reported clinical experience
Madrazo et al (1988, 1990a,b) reported findings in 4 patients with Parkinson's disease who received foetal VM tissue implants from foetuses obtained from spontaneous
Table 1.2. Foetal ventral mesencephalic transplants in man

<table>
<thead>
<tr>
<th>STUDY</th>
<th>METHOD</th>
<th>FOETAL AGE &amp; SITE</th>
<th>No.</th>
<th>DRUGS CHANGED</th>
<th>IMMUNOSUPPRESSION</th>
<th>OUTCOME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Madrazo et al</td>
<td>open microsurgical intra-caudate; tissue pieces</td>
<td>12-14</td>
<td>4</td>
<td>Yes</td>
<td>Yes</td>
<td>improvement over 4 months</td>
</tr>
<tr>
<td>(1988,1990a,b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hitchcock et al</td>
<td>stereotaxic into R caudate; mechanically dissociated tissue</td>
<td>12-19</td>
<td>&gt;12</td>
<td>Yes</td>
<td>No</td>
<td>immediate improvement over 3 months</td>
</tr>
<tr>
<td>(1988,1990)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lindvall et al</td>
<td>stereotaxic into most affected caudate &amp; putamen</td>
<td>6-10</td>
<td>4</td>
<td>No</td>
<td>Yes</td>
<td>no change in 2; marked improvement in patient no.3 over 6 months</td>
</tr>
<tr>
<td>(1988-1990)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freed et al</td>
<td>stereotaxic into most affected caudate &amp; putamen</td>
<td>7</td>
<td>1</td>
<td>No</td>
<td>No</td>
<td>improvement sustained over 1 year</td>
</tr>
<tr>
<td>(1990)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molina et al</td>
<td>open microsurgical intra-caudate; tissue pieces?</td>
<td>6-12</td>
<td>23</td>
<td>Yes</td>
<td>?</td>
<td>improvement over 18 months</td>
</tr>
<tr>
<td>(1989)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The Table summarises the reports of foetal ventral mesencephalic transplants performed in the experimental treatment of Parkinson's disease. The use of different transplantation techniques, location and number of implantation sites, age and number of foetuses, changes in antiparkinsonian drug regimes, and use of immunosuppression, complicate interpretation of these results. In some cases, insufficient details regarding patient selection criteria are given.
abortions. Marked clinical improvement (bilaterally) was noted within 4-8 weeks of surgery, and continued for a further 4 months. Drug regimes were changed perioperatively, and all patients were immunosuppressed. Using tissue from foetuses as old as 18 weeks and stereotaxic implantation into the caudate, Hitchcock et al (1988, 1990) reported symptomatic improvement, which often started immediately after implantation. Drugs were altered perioperatively. Using the criteria that were shown in animal experiments to be optimal for successful grafting of human foetal tissue (Brundin et al 1985b, 1988b, 1989b), Lindvall et al (1988, 1989, 1990) grafted fresh human VM tissue obtained from foetuses of 8-10 weeks' gestation into the putamen and caudate of 4 patients with Parkinson's disease. One of these patients has shown significant motor improvement lasting for up to 8 months, with evidence of surviving graft tissue on positron emission tomography (PET) scanning of $^{18}$F-DOPA uptake. Marked clinical improvement was reported in a patient receiving a human foetal graft without immunosuppressive treatment for up to 1 year after transplant (CR Freed et al 1990). One post mortem study of a patient with 'end-stage parkinsonism', who received implantation of human foetal tissue that had been cryopreserved, revealed no surviving TH-positive cells in the grafted tissue (Redmond et al 1990).

1.3.4.2. Unresolved issues
Although transplantation of human foetal tissue into the brains of patients with Parkinson's disease has already begun, there are many potential problems and unanswered questions. These include ethical issues, practical problems such as obtaining sufficient amounts of foetal tissue (Brundin et al 1990), and limited knowledge regarding factors regulating the growth of the implanted cells. Some of the most pressing issues include, first, the need to identify the optimum site(s) of implantation
of the tissue within the striata in order to achieve maximal symptomatic relief; and, second, the question of whether cells implanted 'ectopically' into the striatum will be affected by the underlying disease process.

Concurrent treatment with L-DOPA
A third pressing issue is the question of whether concurrent treatment with L-DOPA of patients receiving foetal grafts might be harmful to the implanted cells. Ever since its introduction, there has been concern that L-DOPA may be detrimental to surviving DA neurons in the SN of patients with Parkinson's disease (Barbeau 1984; Cohen 1986). Most evidence supporting a possible toxic effect of L-DOPA comes from in vitro work. Graham et al (1978) found that toxic effects of DA to C1300 neuroblastoma cells in vitro may be mediated by auto-oxidation of dopa and DA, giving rise to neurotoxic species such as 6-OHDA and quinones (Figure 1.2A), hydrogen peroxide and free radicals (Figure 1.2B).

The molecular mechanisms responsible for the neurotoxic effects of 6-OHDA, and possibly DA, are related to the ease with which these substances are oxidised at neutral pH (reactions 1-6 in Figure 1.2B). Several potentially cytotoxic compounds are produced in this oxidation process, which is thought to occur mainly in the cell bodies of CA neurons, where the highest concentrations of DA and 6-OHDA are found (Cohen 1986). Normal metabolism of DA results in formation of H₂O₂, which can be cytotoxic in high concentrations (reaction (1) in Figure 1.2B). The initial spontaneous reaction between 6-OHDA and oxygen produces semiquinones and the superoxide radical (reaction (2)). Once superoxide (O₂⁻) is produced it acts as a catalyst to produce H₂O₂ and quinones from dopamine (reaction (3)), and further oxidises H₂O₂ to produce the highly reactive hydroxy radical OH⁻ (reaction (4)). Subsequent reaction of semiquinone with oxygen or O₂⁻ itself leads to formation
Quinones, 6-OHDA and hydrogen peroxide are all produced by oxidation of DOPA and DA (A). Free radicals - superoxide (O$_2^-$) and OH$^-$ - are also produced by oxidation of 6-OHDA and H$_2$O$_2$ (B) (see section 1.2.7.4). All these species are thought to be cytotoxic. The quinones form addition products with sulphydryl groups (SH-) of enzymes and structural proteins in the cytoplasm and axonal membrane (Jonsson 1983; Langston et al 1987). Hydrogen peroxide (H$_2$O$_2$) is an oxidising agent but not very reactive, although it crosses cell membranes easily. It is toxic to many cells, but direct toxicity may only occur at high concentrations; most of its toxicity may be indirect through formation of highly oxidising free radicals (Halliwell 1989). The OH$^-$ radical reacts with almost every molecule found in living cells, including deoxyribonucleic acid (DNA), membrane lipids proteins and carbohydrates (Halliwell 1989), at rates which are limited only by diffusion (Cohen 1986). Cohen (1986) believes that H$_2$O$_2$ and the free radicals O$_2^-$ and OH$^-$ are more important in the neurotoxicity of 6-OHDA than the quinones. There is substantial evidence that superoxide is cytotoxic, but the exact mechanism is unknown (Halliwell 1989).

It is through this series of oxidative reactions that L-DOPA and dopamine may be potentially toxic to dopamine cells.

□□ = potentially cytotoxic species
**Figure 1.2 (A) (adapted from Graham et al 1978)**

TYROSINE

- \( \text{dopa-quinone} \) ➔ leuko-dopachrome ➔ dopachrome

DOPA

- topa ➔ topa-\( \text{p-quinone} \)

DOPAMINE

- dopamine-o-quinone ➔ leuko-aminochrome ➔ aminochrome

- 6-hydroxy-dopamine (6-OHDA) ➔ 6-OHDA-o-quinone ➔ 6-OHDA-p-quinone

- \( \square \) = potentially toxic species

**Figure 1.2 (B) (adapted from Cohen et al 1986)**

1. \( \text{DA} + O_2 + H_2O \rightarrow \text{DOPAC} + NH_3 + H_2O_2 \)
2. 6-OHDA + \( O_2 \) ➔ semiquinones + \( O_2^- + H^+ \)
3. \( \text{DA} + O_2^- + H^+ \rightarrow \text{semiquinone} + H_2O_2 \)
4. \( H_2O_2 + O_2^- \rightarrow OH^- + OH^- + O_2 \)
5. semiquinone + \( O_2 \) ➔ \( H_2O + \text{quinone} + H_2O_2 + OH^- \)
6. semiquinone + \( O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \) (5 and 6 occur spontaneously or are catalysed by superoxide dismutase)
7. \( \text{Fe}^{2+} + H_2O_2 \rightarrow \text{Fe}^{3+} + OH^- + OH^- \)
of quinones and $\text{H}_2\text{O}_2$, together with hydroxy radical $\text{OH}^\cdot$ (reactions (5) and (6)). $\text{H}_2\text{O}_2$ produced in these reactions can form highly oxidising radicals by the Fenton reaction (7). The mechanisms by which $\text{H}_2\text{O}_2$, free radicals and the quinones are thought to produce their toxic effects are outlined in the legend to Figure 1.2.

In addition to the toxic metabolites outlined above, it has been shown that L-DOPA and dopamine, in the presence of ferric iron ($\text{Fe}^{3+}$), can cause death of cultured dorsal root ganglion cells via free radical generation and increased lipid peroxidation (Tanaka et al. 1991). This may be of relevance to patients with Parkinson's disease receiving L-DOPA, where the SN contains elevated levels of ferric iron and lipid peroxide (Dexter et al. 1989).

However, whether L-DOPA or DA are toxic to DA neurones in vivo is still uncertain. There is no convincing evidence that L-DOPA treatment is harmful to DA neurones in the SN or VTA of normal animals (Sahakian et al. 1980; Hefti et al. 1981; Perry et al. 1984). Few studies have addressed the question of L-DOPA toxicity in patients with Parkinson's disease. Yahr et al. (1972) reported that nigral lesions in Parkinson's disease were no greater in L-DOPA treated patients, and there is clinical evidence that L-DOPA treatment does not worsen disability or mortality in Parkinson's disease (Blin et al. 1988). Nevertheless, the effects of L-DOPA treatment on grafted foetal DA cells are uncertain. Foetal DA cells implanted 'ectopically' into the striatum of a foreign host may, by virtue of abnormal location, and possibly also by their immaturity, be more vulnerable to possible toxic effects of L-DOPA than their adult counterparts in the SN and VTA. A separate concern about the administration of L-DOPA to subjects receiving foetal grafts is the effect on graft 'autoreceptors'. Dopaminergic stimulation of autoreceptors located on the nerve terminals and dendrites of SN DA neurones, results in inhibition of
synthesis and release of DA from the terminals (Roth 1984). Autoreceptors may exist on grafted DA neurones (Strecker et al 1987). Conceivably, therefore, autoreceptor stimulation by exogenous L-DOPA (and subsequently DA) may inhibit the ability of grafted cells to synthesise and release DA. In addition, DA agonist stimulation of postsynaptic D-1 DA receptors may interfere with the outgrowth of fibres from developing DA neurones (Lankford et al 1988).

1.4. AIM OF THESIS

The successful application of foetal DA cell transplantation to the treatment of Parkinson's disease will benefit from further experimental work. The aim of this thesis is to investigate whether oral L-DOPA treatment, (administered at a dose which produced plasma levels of L-DOPA similar to those found in patients receiving this drug), is detrimental to the survival of DA cells in rat foetal VM grafts, and to their functional effects, using the unilateral 6-OHDA lesioned model of Parkinson's disease. The functional effects of the rat foetal grafts and any adverse effects due to L-DOPA treatment have been assessed behaviourally, using apomorphine- and (+)-amphetamine-induced rotation and also by studying the effects of grafts and L-DOPA treatment on the autoradiographic density of D-1 and D-2 DA receptors in the striatum. DA cell survival has been studied using tyrosine hydroxylase immunohistochemistry. DA fibre outgrowth has been studied using \(^3\)H-mazindol autoradiography. The glial reaction within and around grafts has been examined.
Chapter 2

MATERIALS AND METHODS
2.1. INTRODUCTION

A summary of the methods used in this thesis is outlined below:

- Production and evaluation of the unilateral 6-OHDA lesion:
  Rats with a unilateral 6-OHDA lesion of the medial forebrain bundle (MFB) were used. Apomorphine- and (+)-amphetamine-induced rotation tests were used to assess the success of the lesion in the first instance. Tyrosine hydroxylase (TH)-like immunohistochemical staining was used as a marker of DA cells. TH-immunostaining was used to verify the lesion by counting the number of TH-positive cells remaining in the substantia nigra (SN) and ventral tegmental area (VTA) on each side.

- Foetal ventral mesencephalic (VM) grafts
  Adult rats were mated on site and foetal tissue of the appropriate gestational age was obtained. Foetal VM tissue was implanted into the striatum of adult rats and functional activity of the grafts was established with apomorphine- and (+)-amphetamine-induced rotation testing. Survival of the grafts was assessed using TH-immunostaining.

- Glial reaction in animals with 6-OHDA lesions, foetal VM grafts and after treatment with L-DOPA and carbidopa:
  The glial reaction in the striata of animals was assessed as a possible indicator of the extent to which the milieu had been disrupted by the various procedures or drug treatments. Glial fibrillary acidic protein (GFA-P) immunohistochemistry was used as a marker for astrocytes.

- Striatal DA fibre density assessed using \(^3\)H-mazindol autoradiography.
  Mazindol is taken up specifically by the catecholamine uptake sites on nerve terminals and dendrites of catecholamine neurons. When used in combination with desmethylinipramine (DMI), an inhibitor of noradrenalin uptake sites, mazindol uptake is limited to DA neurons, and so serves as a specific marker for DA nerve terminals (Javitch et al 1984). This was used to assess both host- and graft-derived DA nerve terminals.
The striatal density of DA D-1 and D-2 receptors. This was assessed using $^3$H-SCH 23390 and $^3$H-spiperone autoradiography.

2.2. DRUGS AND CHEMICALS

6-hydroxydopamine-HBr (Sigma)
Sodium pentobarbital ('Nembutal', Sigma)
Apomorphine-HCl (Sigma)
(+)-Amphetamine-sulphate (Sigma)
L-DOPA (Roche products)
Carbidopa (a-methyl-dopahydrazine, Merck Sharpe and Dohme)
Trypsin (Crude type II, Sigma)
Acridine orange, ethidium bromide (Sigma)
$^3$H-mazindol (Du Pont NEN)
$^3$H-spiperone and $^3$H-SCH 23390 (Amersham)
Anti-tyrosine hydroxylase antibody (Eugene Tech, New Jersey)
Anti-glial fibrillary acidic protein (Dakopatts)
ABC vectastain (Vector labs)
3,4-diaminobenzidine (Sigma)
Isopentane (Fisons)

2.3. SURGICAL TECHNIQUES

2.3.1. Unilateral 6-OHDA stereotaxic lesion of the left medial forebrain bundle (MFB)

Female wistar rats weighing 200-250g at the start of the experiments were used. Rats were anaesthetised with sodium pentobarbital 50mg/kg ip and placed in a stereotaxic frame (Kopf instruments). Ear bars were fixed in position against the tympanic membrane. The nose bar was fixed behind the inner surface of the upper incisors, and raised to 4.5-5.0 mm above the inter-aural line, according to the atlas of Pellegrino et al (1979). The position of the nose bar was adjusted for different rat weights according to Whishaw et al (1977). A midline sagittal incision was made through the scalp to expose the underlying skull. Bregma and the coronal and sagittal sutures were identified. A burr hole approximately 3mm in diameter was created with a dentist's drill over the area to be used in the stereotaxic
injection. A 10μl Hamilton syringe (int. diam 0.13mm; ext. diam 0.47mm) containing 6-OHDA-HBr (8μg as the base dissolved in 4μl 0.9% sterile saline, with 0.1% ascorbic acid w/v) was attached to the vertical instrument carrier. The syringe needle was carefully positioned vertically over bregma and, using the vernier scales, it was moved to the area above the burr hole using the coordinates AP -2.2mm (from bregma), L 1.5mm (to the left of the midline). The needle was lowered until it just touched the dura, which was then punctured with a sterile needle. The syringe needle was lowered 8mm below the level of dura, into the underlying brain. These coordinates correspond to the position of the MPB just rostral to the SN, using the atlas of Pellegrino et al (1979). The solution containing 6-OHDA was injected at a rate of 1μl/min and the needle was left in situ for a further 4 minutes to allow diffusion into the surrounding tissue and to minimise back-leakage up the needle tract, and then slowly withdrawn. The scalp was dried with sterile swabs and closed with Michel surgical clips. Animals were kept in a warm room during recovery from anaesthesia.

An example of the appearance of the SN and VTA several weeks after a unilateral 6-OHDA lesion is shown in Figure 2.1, stained immunohistochemically for TH. This 6-OHDA lesion consistently resulted in loss of >96% of TH-positive cells in the SN ipsilateral to the lesion (compared with the intact side) with a more variable reduction in dopamine cell number in the VTA (range 16-66% of intact side). Routinely, TH-positive cells in the SN and VTA were counted through level -5.8 from bregma, (using the atlas of Paxinos and Watson 1982) as here the SN and VTA are clearly separated by the emerging rootlets of the oculomotor nerve (Figure 2.2).

2.3.2. Preparation of rat foetal VM cell suspensions

2.3.2.1. Timed pregnancies

Foetal brain tissue was obtained from pregnancies resulting from timed matings (the latter according to the methods of Short and Woodnott, 1969). Sexually mature female wistar rats aged 90-110 days were used. Rats have a polyoestrus cycle of 4 days with a gestation
The photomicrograph (2.1A) shows part of a section through the right (intact) SN from an animal receiving a unilateral 6-OHDA lesion of the left medial forebrain bundle, stained for tyrosine hydroxylase (TH). Many TH-positive cells are seen in the intact SN. This is in contrast to the lesioned SN shown in figure 2.1B. TH-positive cells are virtually absent from the SN, but some remaining cells can be seen in the adjacent VTA. Figure 2.1C shows TH staining of a section (40μm) through the intact and 6-OHDA-lesioned ventral tegmental area (VTA) of the same coronal section as that shown in figure 2.1A and B. The animal received a unilateral 6-OHDA lesion of the left MFB, which resulted in incomplete loss of dopamine cells (range 16-66% of the intact side) in the ipsilateral VTA. Many TH-positive cells can be seen in the intact VTA on the right side but cell numbers are markedly reduced in the left VTA.
Figure 2.2 TH staining of a section (40µm) through the intact side of the midbrain taken through level AP -5.8 from bregma (Paxinos and Watson 1982).

At this level the VTA and SN can be seen to be clearly separated by the rootlets of the oculomotor nerve (III N). TH-positive cell counts in the SN and VTA of each side were made in sections taken through this level.
period of 21-23 days. A colony of breeding rats was kept. Oestrus was assessed by performing vaginal smears: a drop of saline was pipetted into and out of the vagina and then transferred to a microscope slide. The oestrus cycle can be divided into 5 stages according to the cellular composition of the vaginal smear (Table 2.1). The time of maximum fertility is oestrus stages II and III, when the vaginal smear consists primarily of cornified epithelial cells. Rats found to be in oestrus stage II or III were placed individually overnight with a male rat in a mesh-bottomed cage with a tray placed underneath. The following morning the presence of a 'copulation plug' in the tray was taken as evidence that coitus had occurred. The plug is a hard yellowish irregular shaped mass about 1/2" long and 1/4" diameter. A vaginal plug does not always indicate a successful pregnancy. The day on which a vaginal plug was found (i.e. the day after mating) was counted as embryonic day (ED) 0.

2.3.2.2. Dissection of foetal VM and preparation of cell suspensions
Rat foetal tissue for transplantation was obtained from timed pregnancies of ED13-16, corresponding to foetal crown-rump lengths (CRL) of 10-13mm in the Wistar rat. Cell suspensions were prepared according to the method of Bjorklund et al (1983a). Sterile instruments and solutions were used. Pregnant rats were anaesthetised using sodium pentobarbital (50mg/kg ip) and a horizontal incision was made in the suprapubic region. A lateral horn of the pregnant uterus was identified and foetuses were removed using fine scissors in groups of 4-5, and placed into a petri dish containing sterile 0.9% saline. Individual amniotic sacs were lifted with forceps, and carefully snipped so that the foetus fell on to a clean microscope slide in a drop of amniotic fluid. The foetal head was severed and placed into a black-bottomed dish containing 0.6% glucose saline and examined under an operating microscope (Zeiss). Using iridectomy scissors (John Weiss) and Dumont no. 5 Watchmakers forceps, the overlying cranium was removed taking care not to traumatise the underlying CNS tissue. The VM was identified in the mesencephalic flexure and cut out into a butterfly shaped piece of tissue using the iridectomy scissors (Figure 2.3). The thin mesenchymal layer on the ventral surface was carefully lifted off.
Table 2.1. Cellular constituents of vaginal smears of sexually mature female rats (taken from Short and Woodnott 1969).

The stages of oestrus as reflected in changes in the cellular constituents of the vaginal smear of rats is shown. Vaginal smears are obtained by pipetting a drop of saline into and out of the vagina. The fluid is then examined microscopically, and the proportion of epithelial cells (with marked nuclei), cornified epithelial cells (large cells paler staining) and leucocytes (small darkly staining cells) are noted. Leucocytes are present at all stages except when the rat is on heat (stages II and III). At the approach of oestrus (stage II) the leucocytes disappear and the smear consists mainly of epithelial cells. When the leucocytes have completely disappeared and the smear consists mainly of cornified epithelial cells (Stage III), the female is on heat. After oestrus, the leucocytes reappear among the cornified cells (Stage IV), indicating the female is no longer on heat.

<table>
<thead>
<tr>
<th>STAGE</th>
<th>VAGINAL SMEAR</th>
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<tbody>
<tr>
<td></td>
<td>Epithelial Cells</td>
</tr>
<tr>
<td>I. Pro-oestrus</td>
<td>+++</td>
</tr>
<tr>
<td>II. Early oestrus</td>
<td>-</td>
</tr>
<tr>
<td>III. Late Oestrus</td>
<td>+</td>
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<tr>
<td>IV. Met-oestrus</td>
<td>+</td>
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<tr>
<td>V. Dioestrus</td>
<td>+</td>
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</table>
Figure 2.3. A diagram of the rat foetal brain as seen under the operating microscope, illustrating important landmarks.

The ventral mesencephalon is outlined by a dotted line opposite the pontine flexure. A butterfly shaped piece of tissue is obtained on dissection of this area.
Pieces of VM were collected in this way from the entire litter (8-14 foetuses) and placed into a dish containing sterile 0.6% glucose saline. Tissue pieces were then transferred into a Durham micro-test tube containing 150μl of 0.6% glucose saline (w/v) and 0.1% (w/v) trypsin (crude type II; Sigma), and incubated at 37°C for 20 minutes (including the time of warming up from room temperature). The trypsin was removed by rinsing 5-6 times using the glucose-saline solution. Finally 0.6% glucose saline was added at a volume equivalent to 10μl per VM piece. The tissue pieces were mechanically dissociated (20-30 strokes) with fire-polished pasteur pipettes of progressively decreasing diameter (1.5 to 0.5mm) to obtain a milky solution. Tissue pieces visible to the naked eye were retained in the suspension, as these had higher cell viability on *in vitro* vital staining than cells in suspension.

2.3.2.3. Assessment of cell viability and concentration in suspensions
All implantation surgery was performed within 4 hours of the trypsinisation step, as this has been shown to be optimal for *in vitro* and *in vivo* viability of rat foetal VM cells (Brundin et al 1985a). At the end of surgical sessions *in vitro* viability of the cell suspensions was assessed using the vital stain acridine orange and ethidium bromide (Sigma; 5μg/ml for each component in 0.9% saline), using a Neubauer haemocytometer. Vital staining with acridine orange and ethidium bromide required fluorescence microscopy with epi-illumination at 390nm (Zeiss fluorescence microscope) with transmitted white light to visualise the haemocytometer chambers. A typical appearance of cells from a suspension 6 hours after the end of a surgical session is shown in Figure 2.4A, with a clump of cells shown in Figure 2.4B (stained with acridine orange and ethidium bromide).

The following method and formulae were used for calculation of cell numbers in the suspension:
Aliquots of suspension were mixed with known volumes of vital stain and transferred at once to the haemocytometer chamber. The total
Figure 2.4. Vital staining with acridine orange and ethidium bromide of a cell suspension prepared from rat foetal ventral mesencephalon. Fluorescence photomicrographs of dispersed cells (A) or a tissue fragment (B) taken from a foetal VM suspension stained (approximately 6 hours after preparation) with the vital stains acridine orange and ethidium bromide. Viable cells stain green, whilst non-viable ones stain orange. In this example, the viability of the dispersed cells is about 65%, whilst most of the cells in (B) appear viable.
numbers of viable (stained green) and non-viable (stained orange) cells were counted within each large square over 10 minutes. The cell concentration in the suspensions was calculated knowing the dilution volumes and the volume in the haemocytometer squares, and the cell viability was expressed as a percent of the total number of cells (see below). Where cell clumps were present, the cell concentration could not be determined. The haemocytometer consists of finely lined large and small squares which are visible when focusing with white light. Each large square of the haemocytometer, with a cover slip in place, represents a total volume of 0.1mm³ or 10⁻⁵cm³. Since 1cm³ is equivalent to 1ml, the cell concentration per ml and % viability are determined as follows:

\[
\text{CELLS PER ML} = \text{average count per square} \times \text{dilution factor} \times 10^4
\]

(where the dilution factor is known from the amount of vital stain added to a given aliquot of cell suspension)

\[
\% \text{ CELL VIABILITY} = \frac{\text{total viable cells}}{\text{total cells (viable and non viable)}} \times 100
\]

### 2.3.3. Stereotaxic implantation surgery

Cell suspensions of rat foetal VM prepared as above were used for grafting into the 6-OHDA-lesioned striatum of rats. Rats were anaesthetised with sodium pentobarbital (50mg/kg ip) and placed in a stereotaxic frame (section 2.3.1.), with the nose bar 4.5-5.0 mm above the inter-aural line. A sagittal incision was made in the scalp and the skull exposed. Bregma was identified and 2 burr holes each 2-3mm in diameter were made with a dentist's drill in the skull overlying the area corresponding to the AP and L coordinates used for implantation. A 15µl glass microsyringe (Scientific glass engineering) with a removable needle of int. diam. 0.2mm and ext. diam. 0.5mm was attached vertically to the instrument holder of the Kopf frame. Foetal VM cell suspension was drawn up into the syringe, and 2µl aliquots were infused at a rate of 1µl/min at each of 3 sites. The syringe needle was moved into position using the vernier scales at the following sites in the denervated striatum (Pellegrino et al 1979): AP +1.8mm from bregma, L +2.5mm from the midline and V 5.0mm below dura (which was first punctured with a
sterile needle); AP +1.0mm, L +3.5mm, and V -7.0mm and -5.0mm below dura. The same cell suspension was used to graft equal numbers of rats in the different groups in any surgical session, and the order of transplantation between groups was alternated, so as to obtain as much uniformity between groups as possible. Animals receiving sham grafts received glucose saline only at the same sites.

2.4. BEHAVIOURAL TESTS USED IN THE ASSESSMENT OF THE 6-OHDA LESION, FOETAL VM GRAFT AND L-DOPA TREATMENT

2.4.1. Spontaneous rotation
Spontaneous rotation was observed informally when the animals were in their cages including those periods when animals received L-DOPA and carbidopa in their drinking water.

2.4.2. Apomorphine-induced rotation and stereotypy
An initial screen for the completeness of the unilateral 6-OHDA lesion of the MFB was performed 1 week after lesion surgery using apomorphine 0.5mg/kg sc or 0.05mg/kg sc (Sigma; dissolved in sterile water). Rats were first habituated in test boxes (perspex rectangular boxes 260cm (W) X 380cm (L) X 180cm (H)) for 30 mins. Immediately after drug injection (apomorphine 0.5mg/kg sc or 0.05mg/kg sc) circling rates were counted visually for one minute periods at 6-10 minute intervals over 30 minutes. The mean number of rotations obtained per minute over this period was calculated for each rat. Only rats showing a mean of >6 contralateral rotations per minute over the test period were included for further study, this being consistent with a near-complete lesion of the ipsilateral SN (Ungerstedt 1971c; Hefti et al 1980).

A preliminary dose response curve revealed that apomorphine 0.05mg/kg sc was the smallest dose capable of eliciting marked contralateral rotation in animals with unilateral 6-OHDA lesions (Figure 2.5A), whilst a dose of 0.5mg/kg sc resulted in marked contralateral rotation in addition to apomorphine-induced stereotypy (AIS), in the form of licking and biting. A typical time course of the rotational response of rats with a 6-OHDA lesion, following administration of apomorphine (0.05mg/kg sc) over 30 minutes is shown in Figure 2.5B.
Figure 2.5. Apomorphine-HCl dose response curve (A) and time course (B) of contralateral rotation following administration of apomorphine to rats with a unilateral 6-OHDA lesion.

All animals had received a unilateral 6-OHDA lesion of the MFB two weeks previously. Rotations were counted visually for 1 minute every 6 minutes over a 30 minute (A) or 60 minute (B) period.

(A) The apomorphine dose-response curve was obtained using at least 6 animals for each dose. The values shown are the mean (+1 SEM) number of contralateral rotations per minute over a 30 minute period. Doses ranged from 0.0625mg/kg sc to 0.75mg/kg sc. A dose of 0.05mg/kg sc was the minimum dose producing significant contralateral rotation, whereas a dose of 0.5mg/kg sc produced marked stereotypy in some animals in addition to contralateral rotation.

(B) The time course of the rotational response following administration of apomorphine (0.05mg/kg sc) in drug-naive rats (n=6) is shown. Values shown are mean (+1 SEM) number of contralateral rotations per minute. Marked contralateral rotation generally began 6-8 minutes after drug administration, and peaked at 15-25 minutes. Subsequently high rates of contralateral rotation continued for the rest of the 30 minute test period. Positive scores are contralateral to the lesioned side.
A

Mean no. contralateral rotations/minute vs. Dose apomorphine (mg/kg sc)

B

Mean no. contralateral rotations/minute vs. Time (minutes)

73
The stereotyped response of rats to apomorphine HCl (0.5mg/kg sc) was recorded. AIS was assessed 15 minutes after drug administration. Animals showing intense stereotypy (i.e. continuous licking, gnawing or biting) were noted.

The rotational and stereotyped response to apomorphine was reassessed prior to sham or foetal graft surgery and prior to commencing L-DOPA treatment to ensure that there had been no spontaneous reduction in rotation rates, and again at various times after sham- or foetal graft surgery and L-DOPA treatment.

2.4.3. (+)-Amphetamine induced rotation
One-two weeks after 6-OHDA lesion surgery, and only after successful testing with apomorphine, rotation was assessed over a 60 minute period following administration of (+)-amphetamine sulphate (Sigma; 5mg/kg ip dissolved in sterile water). This dose was chosen as it was the smallest dose consistently producing marked ipsilateral rotation in rats with unilateral 6-OHDA lesions, as shown in Figure 2.6A. Rats were first habituated for 30 minutes in the perspex test boxes. Immediately following administration of (+)-amphetamine (5mg/kg ip), rotations were counted for 1 minute periods at 6 minute intervals for the first 30 minutes after injection and then at 10 minute intervals for the remaining 30 minutes. Only rats showing a mean score of >8 ipsilateral turns/minute over the 60 minute period were included for further study. Rats showing this degree of (+)-amphetamine-induced ipsilateral rotation have >99% depletion of dopamine in the ipsilateral striatum (Schmidt et al 1982, 1983). A typical time course of the rotational response of rats with a 6-OHDA lesion following administration of (+)-amphetamine (5mg/kg ip) is shown over 60 minutes (Figure 2.6B). (+)-Amphetamine-induced rotation was reassessed prior to sham or foetal graft surgery, and at various times after such surgery.

2.5. L-DOPA AND CARBIDOPA TREATMENT
L-DOPA (Roche products, UK) plus carbidopa (α-methyl-dopahydrazine, Merck Sharp and Dohme, UK) were administered in drinking water provided as the only source of fluid at target doses of 200mg/kg/24 h
Figure 2.6. (+)-Amphetamine sulphate dose response curve (A) and time course (B) of ipsilateral rotation following administration of (+)-amphetamine sulphate to rats with a unilateral 6-OHDA lesion.

All animals (n=6) had received a unilateral 6-OHDA lesion of the medial forebrain bundle 2 weeks earlier. Rotations were counted visually for 1 minute every 6 minutes for the first 30 minutes, and then every 10 minutes for the remaining 30 minutes.

(A) For the (+)-Amphetamine-sulphate dose response curve at least 6 rats were used for each dose. Doses ranged from 0.0625mg/kg ip to 0.75mg/kg ip. Values shown are the mean (+1 SEM) number of ipsilateral rotations per minute over a 60 minute period. A dose of 5mg/kg ip was the smallest dose consistently producing marked ipsilateral rotation.

(B) An example is shown of the time course of the rotational response following administration of (+)-amphetamine (5mg/kg ip) in drug-naive rats. Values shown are mean (+ 1SEM) number of ipsilateral rotations per minute over a 60 minute period. Animals generally began rotating 5-8 minutes after injection and peaked ~18 mins after injection. High rates of rotation continued for the rest of the 1 hour period.
and 25mg/kg/24h respectively. Treatment was commenced 18-24 hours after foetal or sham graft surgery. Drug intake was assessed by monitoring the volume of water drunk (twice weekly, in rats housed in groups of 6) and body weights (weekly). Drug concentrations were altered accordingly each week. Leakage of water from the bottles was prevented by use of ball bearings in the teats of all bottles. L-DOPA and carbidopa were dissolved in a minimum volume of 2N HCl, made up to the desired volume with distilled water, and adjusted to a final pH of 4.5-5.0 using 2N NaOH. Ascorbic acid (0.1% w/w) was added and bottles were protected from the light as precautions against oxidation of L-DOPA. Fresh drug solutions were prepared every 3-4 days. Such a regime results in stable concentrations of L-DOPA and carbidopa for a minimum of 5 days (S. Rose, 1991).

Preliminary studies in a group of rats (n=6) with a unilateral 6-OHDA lesion showed that this drug dosage regime resulted in plasma levels of L-DOPA of 0.895±0.306 μg/ml at the end of the dark cycle, as measured by High Performance Liquid Chromatography (HPLC) (section 2.7). These plasma levels are slightly higher than mean peak plasma levels found in patients receiving these drugs (Hare et al 1973, 0.6μg/ml; Marion et al 1986, 0.1-0.6μg/ml). An oral regime was chosen in preference to an intra-peritoneal (ip) for the following reasons: first, it is similar to the route of administration used clinically, and different routes of administration of L-DOPA may have different effects on behavioural function (Juncos et al 1989). Second, ip injection of rats with L-DOPA (100mg/kg) was shown to result in peak plasma levels that were 5-6 times as high as those achieved when the same dose was given orally (Hare et al 1973).

2.6. HISTOLOGICAL AND IMMUNOHISTOCHEMICAL PROCEDURES

2.6.1. Preparation of brains
Unless animals had died unexpectedly, brains were fixed by perfusion with 4% paraformaldehyde in 0.1M Phosphate buffered saline (PBS), pH 7.4. Animals were anaesthetised with sodium pentobarbital 50mg/kg ip and placed on a wire tray over a sink with running water in a fume cupboard. A transverse horizontal incision was made through the abdominal wall just beneath the costal margin. The descending aorta was clamped. The xiphoid process was raised with blunt forceps, and
the diaphragm was carefully cut away from its insertion to the anterior costal margin. Using a strong pair of blunt scissors, the ribs were cut vertically up to the axilla of each side and the anterior flap of the thoracic cage so produced was lifted to expose the underlying lungs and heart. The left ventricle was identified, and a 'butterfly' (needle gauge 21) connected to a perfusion pump (Cole Parmer Instruments, Chicago) was inserted into the left ventricle and clamped gently in position. Immediately after this the right atrium was incised. Animals were first perfused with 100ml of ice cold PBS (0.1M, pH 7.4) followed by 400ml of ice cold 4% paraformaldehyde in 0.1M PBS, over 15 minutes. The skull bones overlying the cortex were removed with bone cutters and the brain was carefully lifted out with a rounded spatula. Brains were post-fixed in the same fixative for a further 24-48 hours at room temperature. Brains from animals which had died unexpectedly were, if possible, fixed by immersion in 4% paraformaldehyde in 0.1M PBS. Brains which were to be cut on a cryostat after freezing were immersed in a cryoprotectant solution containing 20% dextrose saline for 24 hours after perfusion.

Brains were sectioned either on a vibratome (Biorad instruments; 40-50 µm sections) or on a cryostat (Bright instruments) at -20°C (20µm sections). Coronal sections were taken through the entire striatum, and through the midbrain (to include the SN and VTA). Parallel sections were stained for cresyl violet, tyrosine hydroxylase, glial fibrillary acidic protein or serotonergic immunohistochemistry. The sections allocated for serotonin staining were given to a colleague, and those results do not form part of this thesis.

2.6.2. Slide subbing

Glass microscope slides were dipped for 5 seconds in a staining dish containing 200ml 'subbing solution' (1 g gelatine dissolved at 60°C in 200ml distilled water containing 0.1g Crome Alum).

2.6.3. Cresyl violet staining

Brain sections which had been mounted on gelatin-coated slides and left to dry for 24 hours were stained in cresyl violet (0.1% solution w/v; RA Lamb) as shown in Table 2.2.
2.6.4. Immunohistochemistry

Immunostaining for tyrosine hydroxylase (TH) and glial fibrillary acidic protein (GFAP) was performed according to a modified Sternberger technique (1986), using the avidin biotin complex method (ABC vectastain; Vector Labs) to improve the sensitivity of immunostaining (Hsu et al. 1984). The chromogen in both cases was 3,4-diaminobenzidine (DAB; Sigma). TH-like immunoreactivity was used as a marker of DA cells (referred to here as TH-positive cells). In all staining sessions, specificity of the immunostaining method was ensured by including a known positive 'test' tissue (i.e. sections containing the SN for TH, and sections containing ependyma for GFAP), and by obtaining negative staining of the test tissue when the primary antibody was omitted. Primary antibody was diluted at the maximum dilution compatible with good positive staining.

Free-floating coronal sections of striatum or midbrain that had been perfusion fixed (section 2.6.1.) were rinsed in 0.1M PBS, pH 7.4, three times followed by immersion in 0.3% hydrogen peroxide in PBS for 30 minutes to block endogenous peroxidase activity. Sections were then rinsed twice in PBS and incubated in 20% normal goat serum (NGS) in PBS for 60 minutes. Sections were rinsed in PBS containing 1% NGS (w/v), and 0.05% Triton X-100 to improve penetration of the primary antibody through cell membranes. Primary antibody (either rabbit anti-tyrosine hydroxylase, Eugene tech, New jersey, diluted at least 1:1800 in PBS; or rabbit anti-GFAP, Dakopatts, diluted at least 1:500) was applied to the sections for 2 hours at room temperature and for 48 hours at 4°C (agitated throughout), after which sections were rinsed in PBS containing 1% NGS. The ABC method was used to locate the primary antibody: sections were incubated in biotinylated goat anti-rabbit IgG for 30 minutes, followed by 2 rinses in PBS. A complex of avidin and biotinylated peroxidase was added and incubated for 1 hour. Sections were rinsed in PBS, followed by TRIS-HCL buffer (pH 7.4 0.05M), and then pre-incubated with DAB (0.05% in TRIS buffer) for 5 minutes. Hydrogen peroxide was added to achieve a final concentration of 0.01%, and incubated for 6 minutes. The reaction was stopped by removing the DAB-hydrogen peroxide and adding TRIS-HCl buffer. DAB solution (a possible carcinogen) was inactivated with sodium hypochlorite. Sections
Table 2.2. Protocol for cresyl violet staining

<table>
<thead>
<tr>
<th>Staining dish no.</th>
<th>Solution</th>
<th>Staining Time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Histoclear</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Histoclear</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>100% ethanol</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>100% ethanol</td>
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</tr>
<tr>
<td>5</td>
<td>95% ethanol</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>95% ethanol</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>70% ethanol</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>70% ethanol</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>Dist. water</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>0.1% cresyl violet</td>
<td>2-5</td>
</tr>
<tr>
<td>11</td>
<td>Dist. water</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>Dist. water</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>70% ethanol</td>
<td>5</td>
</tr>
<tr>
<td>14</td>
<td>95% ethanol</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>100% ethanol</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>Histoclear</td>
<td>5</td>
</tr>
</tbody>
</table>

Slide-mounted sections were moved through the series of staining and dehydrating dishes for the allotted times. At the end of the series, sections were covered with mounting medium (DEX) and a coverslip placed on top. Cresyl-violet staining yields a purple colour for nissl substance, a lilac colour for microglia, and the background remains colourless.
stained immunohistochemically or with cresyl violet were mounted on gelatin coated slides and dehydrated through a series of alcohols. Sections were covered with mounting medium (DPX; Sigma) and coverslips placed in position.

2.6.5. Cell counting and graft volume estimation

2.6.5.1. Tyrosine hydroxylase stained sections
For the SN and VTA, TH-positive cells were counted separately on the intact and lesioned side according to the method of Konigsmark (1970). The long and short axes of cells were measured in a representative sample of cells from each animal using the eye piece micrometer, which was calibrated against the stage micrometer (Leitz microscope). At least three sections taken at the level of A-5.8 from bregma (Paxinos and Watson 1982) were selected for each animal (see Figure 2.2). At this level the SN and VTA are clearly separated by the emerging rootlets of the oculomotor nerve. Mean cell numbers were obtained for each side for each animal. The SN and VTA in each section were systematically examined using an eye-piece reticule moving from lateral to medial over both structures. No attempt was made to estimate the total number of cells in these nuclei.

For the foetal grafts in the striatum every 3rd or 4th coronal section through the striatum was examined and all TH-positive cells counted using the method described by Konigsmark (1970). The position of the graft within the striatum was noted. Each section was counted twice. Cross-sectional areas of the grafts were measured with the eye piece reticule. The graft volume was calculated from the sum of the cross sectional areas in all sections and the section thickness. The long and short axes of 10 cells from the dorsal region of each graft were measured, and a mean value for cell size for each animal was obtained.

2.6.5.2. Glial fibrillary acidic protein stained sections
A semi-quantitative estimate of the density of GFAP positive cells in striatal sections was made as follows. The number of GFAP-positive cells per high power field was counted in the right (intact) and left striata in each of 3 positions, as shown in Figure 2.7. An index of
Figure 2.7. Diagram showing the positions in the left and right striatum at which GFAP-positive cells were counted.

The mean number of cells found per high power field (magnification X 320) in each of these positions for at least 5 sections was obtained. An index of the overall glial reaction in each striatum was obtained from a mean value of all 3 positions combined. Glial cell density per high power field within the grafts (g) was also assessed.
the overall glial response within each striatum was obtained by calculating the mean density for the 3 positions combined in all sections studied. A minimum of 5 sections spanning the A-P extension of the graft or sham graft placements were studied for each animal. In animals receiving foetal grafts the mean glial density per high power field within each graft was calculated.

2.7. BIOCHEMICAL ASSESSMENT: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MEASUREMENT OF L-DOPA AND ITS METABOLITES IN STRIATUM AND PLASMA

This was performed according to the principles outlined by Marsden and Joseph (1987).

2.7.1. Tissue preparation

(a) Plasma

Blood was collected from the neck stump into 5ml heparinised tubes (Sarstedt Ltd, UK) from rats killed by a cervical dislocation and decapitation. The blood was centrifuged in a Sorvall RC-3B refrigerated centrifuge at 1,800g for 30 minutes at 4°C to separate the plasma. Plasma was removed and added to an aliquot of a solution containing dihydroxybenzylamine (DHBA) as the internal standard, in 0.4M perchloric acid containing 1mM ethylenediaminetetra-acetic acid (EDTA) and 0.5mM Na₂S₂O₅ ('homogenising acid') in a 1:1 volume:volume ratio (Rose et al 1989). The samples were mixed using a whirlimixer (Fisons Scientific Equipment Ltd), allowed to stand on ice for 10 minutes to ensure deproteinisation, and centrifuged in a Sorvall RC-5B refrigerated centrifuge for 10 minutes at 1,800g at 4°C. Aliquots (10-50μl) of the supernatant were used for High Performance Liquid Chromatography (HPLC) analysis of L-DOPA and its metabolites.

(b) Brain tissue

Rats were killed by stunning and decapitation. The cranium was opened and the brain was removed and placed on filter paper (Whatman No. 1, Fisons Scientific Equipment Ltd), moistened with ice-cold 0.9% saline. The filter paper was placed on a petri dish cooled on ice. The meninges were removed, and the olfactory tubercle and nucleus accumbens ('limbic areas') were dissected from the ventral surface of the brain using a pair of fine watchmakers forceps. The brain was sectioned by two dorso-ventral cuts made at 3.8mm and 1.2mm.
(Pellegrino et al 1979), and striatal tissue was dissected from the slice. The right and left limbic areas and right and left striata were weighed individually and kept separately for each animal and were placed into eppendorf tubes (1.5ml) (Sarstedt Ltd), frozen on dry ice and stored at -70°C until assayed for biogenic amines. On the day of assay, the tissue was thawed on ice and homogenised in 20 volumes of homogenising acid containing DHBA as internal standard (300μl for limbic areas and 500μl for striatum) by sonication in a Microson ultrasonic tissue disruptor (Heat Systems Inc. USA). An aliquot of the resulting homogenate was then added to an aliquot of a solution of DHBA in a 9:1 volume:volume ratio. The final concentration of DHBA was 200ng/ml for homogenates of striatum, and 100ng/ml for homogenates of olfactory tubercle and nucleus accumbens. The homogenates were centrifuged at 30,000 g for 10 minutes at 4°C in a Sorvall RC-5B refrigerated centrifuge. Aliquots (50-100μl) of the resultant supernatant were used for HPLC analysis of tissue monoamines and their metabolites (section 2.7.2.)

2.7.2. HPLC measurement of L-DOPA and its metabolites using electrochemical detection (ECD)

Tissue dopamine and its metabolites were assayed by the method of Wagner et al (1979). Reversed-phase ion-pair chromatography was performed on a Spherisorb ODS 2, 5μm particle size HPLC column (0.46 x 25 cm; Phase Separations Ltd, UK). The mobile phase contained 0.1M Na₂HPO₄, 15-18% methanol, 6-7mM octane sulphonic acid (the ion-pairing agent) and 1M EDTA, pH 2.9-3.2 adjusted with 3M phosphoric acid. It was pumped at a flow rate of 1 ml/min by a Waters 510 pulse-free pump at a pressure of no more than 3500 psi. Samples of standard solution (section 2.7.4), tissue homogenate, or plasma were injected using a 8780XR automated sampler (Spectra Physics Ltd, UK) fitted with a Rheodyne injection valve and a 100μl sample loop. An electrochemical flow cell was used as the detector component (ECD) for liquid chromatography of catecholamines (Kissinger et al 1973). The amount of analyte in each injection was measured using a BAS LC3A amperometric detector with a thin layer electrochemical cell fitted with a glassy carbon working electrode and a Ag/AgCl reference electrode set at a potential of +0.65 to +0.7V. The applied potential used for the analysis of samples was between 0.65 and 0.7V.
as all components used in the standards were detected in this range (S. Rose, 1991). Integration of the signal from the detector was performed using a 4270 computing integrator (Spectra Physics Ltd, UK). The column effluent passes over the anode surface of the detector flow cell, to which any compound which is electro-oxidisable at the chosen potential can lose one or more electrons and become transformed into an oxidised reaction product. For the catecholamines this oxidation consists of a two electron reaction to yield an O-quinoid structure (Marsden and Joseph 1987). The composition of the mobile phase, especially the sodium salt concentration, affects the detector response such that a minimum concentration of 0.05M NaHPO₄ must be used to achieve maximum detector response. A concentration of 0.1M NaHPO₄ was used.

2.7.3. Calibration of the HPLC-ECD system

The HPLC-ECD system was calibrated with a single standard solution equal in concentration to the internal standard concentration in the homogenate. During the analysis of L-DOPA and its metabolites in supernatants prepared from rat tissues, the system was calibrated every 6-8 samples, and the record of the calibration updated. This accounted for any changes in sensitivity of the electrode and changes in the separation capacity of the column over the period of analysis.

On the day of the assay, an aliquot (1.5ml) of a standard solution containing L-DOPA, 3-CMD, dopamine, DOPAC, 5-HIAA, 5-HT and HVA (200μg/ml) was thawed on ice (having been stored at -70°C). Serial dilutions were prepared using homogenising acid giving concentrations of the analytes in the solution of between 0.5 and 1500ng/ml. Linearity of the HPLC system was demonstrated by injecting 50μl aliquots of these standard solutions over the concentration range 0.5ng/ml to 1500ng/ml. Standard curves of peak height ratio (PHR) of the analyte to the internal standard (DHBA), were plotted against analyte concentration and analysed using least squares regression (Figure 2.8). The system proved to be linear over this concentration range (r>0.97 for all analytes). The linearity of the system was demonstrated at the beginning of the assay of a tissue, when a new column was used, and after the electrode had been polished to remove accumulated oxidation products from its surface.
Figure 2.8. Sample standard curves for (A) L-DOPA, (B) 3-CMD, (C) dopamine, (D) DOPAC and (E) HVA, used to calibrate the HPLC system.

The standard curves were prepared by injecting aliquots (50μl) of prepared standard solutions containing L-DOPA, 3-CMD, dopamine, DOPAC and HVA over the concentration range 0.5-1500ng/ml, dissolved in homogenising acid. All solutions contained DHBA at a concentration of 200ng/ml. Aliquots (50μl) of standard solutions were injected onto the HPLC and the peak height (PH) ratio (PHR) of the analyte to DHBA (PH analyte/PH DHBA) was plotted against analyte concentration. Curves were analysed by least squares regression. All were linear over the concentration range 0.5-1500ng/ml (r=0.99, p<0.001).
2.7.4. Recovery from tissue homogenates

Control tissue homogenates of cerebellum and plasma (as prepared in section 2.7.1.) were 'spiked' with standard solution containing L-DOPA, dopamine, DOPAC, HVA, 3-O-MD and DHBA (1000ng/ml) in a 9:1 volume:volume ratio to give a final concentration of 100ng/ml. Samples were analysed for levels of the biogenic amines and their metabolites as described in section 2.7.2. Tissue homogenates of cerebellum and plasma were mixed with a standard solution containing DHBA alone to assess endogenous levels of biogenic amines and metabolites. L-DOPA, dopamine, DOPAC, HVA and 3-O-MD had a recovery of 90% or greater from brain and plasma (Figure 2.9). No compensation for recovery was therefore required.

2.7.5. Calculation of results

The integrator of the HPLC-ECD system calculated the concentration of the analytes in the homogenate using a peak-height (PH) ratio with the internal standard, using the following formula:

\[
\text{Amount in sample (ng/ml)} = \frac{\text{PH(analyte)} \times \text{conc STD} \times \text{PH (STD DHBA)}}{\text{PH (sample DHBA)} \times \text{diln. fact} \times \text{PH (STD analyte)}}
\]

(PH=peak height; diln. fact=dilution factor incurred when adding the solution of DHBA to the homogenate; STD=standard used to calibrate the HPLC system).

For plasma and homogenate samples this gave the final concentration in ng/ml.

Typical sample chromatograms for the standard solution, and for the homogenates prepared from the intact and 6-OHDA-lesioned striatum are shown in Figure 2.10.
Figure 2.9. Recovery of L-DOPA, DA, DOPAC, 3-OHD and HVA from supernatants of homogenates prepared from (A) cerebellum and (B) plasma of normal rats.

Tissues were prepared for HPLC analysis as described in section 2.7.1. In samples with added analyte concentrations an aliquot (50µl) of a standard solution containing L-DOPA, dopamine, DOPAC, 3-OHD, HVA and the internal standard DHBA was added to the tissue homogenate or plasma in place of the aliquot of the solution of DHBA alone. The final concentration of the analytes and the internal standard was 100ng/ml for cerebellum and 500ng/ml for plasma. The samples were analysed for levels of each analyte as described in section 2.7.2. The HPLC system was calibrated with an aliquot (50µl) of the standard solution containing all the analytes (1/10 concentration of the standard solution added to the homogenates). Recovery was calculated by subtracting the basal concentration of the analyte in the tissue supernatant from the concentration in the tissue supernatant after addition of the standard solution. The resulting concentration was divided by the original concentration of the standard solution used to calibrate the HPLC system.

Values are expressed as the mean (± 1 SEM) % of the standard (100ng/ml for cerebellum and 500ng/ml for plasma) used to calibrate the system. Concentrations were calculated using the ratio of the peak height of sample:peak height internal standard (PHR) (see section 2.7.10). All chromatography was performed at 10°C to prevent sample degradation during storage in the autosampler.
Figure 2.10  Sample chromatograms from aliquots (50μl) of standard solution (A) and supernatants from homogenates of intact (B) and 6-OHDA-lesioned (C) rat striatum.

The HPLC system consisted of a Spherisorb ODS 2 (5μm particle size, 25.0 X 0.4 cm ID) HPLC column. The mobile phase contained 0.1M NaH2PO4, 6.5mM octane sulphonic acid, 1mM EDTA, 17% (v/v) methanol, pH 3.1. The flow rate was 1ml/min.

Chromatogram (A) represents an injection of an aliquot (50μl) of a standard solution containing 100ng/ml of NA, L-DOPA, DHBA, dopamine, 3-O-MD, DOPAC, 5HTAA, 5HT and HVA in 0.4M perchloric acid containing 1mM EDTA and 0.5mM Na2S2O3. The peaks corresponding to the analytes are shown. Chromatogram (B) represents an injection of an aliquot (50μl) of the supernatant of a homogenate of the intact striatum of a rat. Peaks for dopamine and its metabolites DOPAC and HVA are present. In contrast, in chromatogram (C), which represents an injection of an aliquot (50μl) of the supernatant of a homogenate of the 6-OHDA-lesioned striatum of a rat, there is a complete absence of the peaks for DA, HVA and DOPAC.
DOPAC

\[ \text{c} \to \text{HVA} \to \text{HIAA} \to \text{DA} \to \text{DHBA} \]

5nA

10 min

A

B

C

DHBA

DOPAC

HIAA

HT

HVA

92
2.8. AUTORADIOGRAPHY OF $^3$H-MAZINDOL, $^3$H-SPIPERONE AND $^3$H-SCH 23390 TO STRIATAL SLICES

2.8.1. Tissue preparation

Female wistar rats (Bantin and Kingman) were anaesthetised with pentobarbital (50mg/kg ip) and perfused transcardially (see section 2.6.1) with 300 ml ice cold 5% dextrose saline. Brains were removed and 'snap' frozen in isopentane (Fisons) and mounted on cryostat chucks. Coronal sections (20µm) through the entire striatum were cut at -20°C using a cryostat (Bright instruments). Sections were thaw-mounted in duplicate onto subbed slides with alternate sections allocated to slides for Total (T) or Non-specific (NS) binding. Sections were dried in a stream of cool air. Sections were frozen at -20°C (< 1 week) until binding studies were carried out.

2.8.2. Preliminary biochemical studies for $^3$H-spiperone binding to striatal slices.

Preliminary tissue swabbing studies were undertaken to determine the kinetic and equilibrium characteristics of $^3$H-spiperone and $^3$H-SCH 23390 binding to striatal slices, according to the method of Kuhar (1985).

For saturation studies, sections were incubated for 1 hour at room temperature in 50 mM TRIS-HCl buffer (pH 7.4) containing 120 mM NaCl (Theodorou et al 1980) and $^3$H-spiperone (specific activity 111µCi/nmol, Amersham) at concentrations ranging from 0.15-2.9nM (the 'standards'). Non-specific binding was defined with haloperidol $10^{-5}$M and 5HT$_2$ sites were blocked with ketanserin $10^{-5}$M. Incubation was terminated by rinsing the sections for 5 minutes each in 2 baths of TRIS buffer at 4°C. Sections were then dipped in cold distilled water for 2 seconds and dried in a stream of cool air. A typical saturation curve is shown in Figure 2.11A, and Scatchard transformation (Scatchard 1949) of the data is shown in Figure 2.11B.

Association of $^3$H-spiperone to striatal slices was determined by incubating sections in TRIS buffer containing 1nM $^3$H-spiperone at room temperature for times ranging from 5-90 minutes. Non-specific binding was defined with haloperidol $10^{-5}$M and 5HT$_2$ sites were
blocked with ketanserin $10^{-5}$M. Association was terminated by rinsing sections in 2 troughs of ice-cold buffer (5 minutes each), followed by a 2 second dip in distilled water. Sections were dried in a stream of cool air. The time course of association is shown in Figure 2.12, and a pseudo-first order plot of association is shown in the inset.

Dissociation of $^3$H-spiperone from striatal slices was determined by first incubating sections for total and non-specific binding to equilibrium (1 hour) at room temperature in TRIS buffer containing 1nM $^3$H-spiperone. Dissociation was initiated by transferring the sections into a trough (4 sections per trough) containing an infinite dilution (300ml) of buffer at room temperature. Buffer was renewed every 20 minutes. Sections were rinsed in buffer for times ranging from 5-60 mins. Sections were then dipped in ice cold distilled water and dried in a stream of cool air. The time course of dissociation is shown in Figure 2.13, and a first order plot of the time course is shown in the inset.

For the association and dissociation experiments, an incubating concentration of 1nM $^3$H-spiperone was chosen as this was close to the equilibrium dissociation constant (Kd) obtained from Scatchard studies (Figure 2.11B), and has been used by others in similar studies (Neve et al 1984).

2.8.3. Preliminary biochemical studies for $^3$H-SCH23390 binding to striatal slices

Preliminary studies were performed according to the method of Savasta et al (1987). For saturation experiments, sections were incubated to equilibrium for 90 minutes at room temperature in 50 mM TRIS-HCl buffer (pH 7.7) containing 120mM NaCl, 5mM KCl, 2mM CaCl$_2$ and 1mM MgCl$_2$ and $^3$H-SCH 23390 at concentrations ranging from 0.1 - 4.0nM. Non-specific binding was defined with CIS-flupenthixol $10^{-7}$M. Following incubation, sections were rinsed twice consecutively (5 minutes each) in buffer, and then dipped (2 seconds) in distilled water and dried in a stream of cool air. A typical saturation curve of $^3$H-SCH 23390 binding to striatal sections is shown in Figure 2.14A and Scatchard transformation of the data is shown in Figure 2.14B.
Figure 2.11. Saturation binding of $^3$H-spiperone to striatal slices (A) and Scatchard transformation (B) of the specific saturation binding data for $^3$H-spiperone.

A. Rat forebrain sections (20μm) were incubated with $^3$H-spiperone (0.15-2.9nM) for 1 h at room temperature. Total binding (○), specific binding (■) and non-specific (NS) binding (□) are shown in counts per minute (CPM). NS binding was linear throughout the range of concentrations, and in the linear part of the specific binding curve, NS binding was never greater than 25% of total binding. Saturation of specific $^3$H-spiperone binding occurred at about 1.2nM.

B. Scatchard transformation of the specific saturation binding data for $^3$H-spiperone (data derived from Figure 2.11A).

Scatchard transformation revealed a Kd of 1.1 (obtained from the gradient of the plot) and Bmax of 58.1 fmols/mg tissue (obtained from the y intercept).

Key: B=ligand specifically bound; F= free ligand.
Figure 2.11

A

CPM

3H-spiperone (nM)

B

y = 58.165 - 1.1796x  R^2 = 0.899
Figure 2.12. Time course of association of $^3$H-spiperone (specific binding) to rat striatal sections (20µM) incubated in buffer containing 1nM $^3$H-spiperone for times ranging from 5-90 minutes.

Association was terminated by rinsing the sections in ice cold buffer. Equilibrium occurred after about 1 hour of incubation. The inset shows a linear pseudo-first order plot of the association time course, consistent with the binding reaction obeying simple mass action kinetics (obtained using the equations of Weiland and Molinoff, 1981).
Figure 2.13. Time course of dissociation of $^3$H-spiperone (specific binding) from striatal slices.

Sections were first incubated to equilibrium (i.e. for 60 minutes) in buffer containing $1\text{mM}^3$H-spiperone. Dissociation was initiated by washing sections for times ranging from 5-60 mins in an 'infinite dilution' of buffer (~300 mls for 4 sections, renewed every 20 mins). A linear first order plot of the dissociation time course, consistent with the reaction obeying simple mass action laws, is shown in the inset.
The rate of association of $^3$H-SCH 23390 to striatal slices was determined by incubating sections for total and non-specific binding in buffer containing 1.0nM $^3$H-SCH 23390 with CIS-flupenthixol $10^{-7}$M to define non-specific binding for times ranging from 5-150 minutes. 1.0nM $^3$H-SCH 23390 is approximately equal to the value for the equilibrium constant $K_d$ obtained from Scatchard analysis of saturation studies (see Figure 2.14B) and has been used by others in similar studies (Savasta et al 1987). Association was terminated by rinsing sections in buffer and distilled water as described above. Sections were dried in a stream of cool air. The time course of association is shown in Figure 2.15, and a pseudo-first order plot of the time course is shown in the inset. For dissociation (Figure 2.16) sections for total and non-specific binding were first incubated to equilibrium (90 minutes) in buffer containing 1.0nM $^3$H-SCH23390. Dissociation was initiated by transferring sections into 300ml buffer for wash times ranging from 5-60 mins. At the end of each wash time sections were rinsed twice in buffer and dried in a stream of cold air. The time course of dissociation is shown in Figure 2.16, and a first order plot is shown in the inset.

2.8.4. Scintillation counting of radioactivity bound to striatal slices

For all preliminary experiments described in sections 2.8.2 and 2.8.3, radioactivity in the standard incubating solutions at the start of the experiments, ligand bound in the sections and 'free' ligand remaining in the incubation troughs was measured in vials in a scintillation counter (Packard Instruments). For the determination of ligand in the standards and free ligand remaining in the troughs, a 50pl aliquot was taken from each concentration, scintillation fluid (5ml) was added and the vial gently shaken. For ligand bound in the sections, the cortex of each section was first scraped off the slide with a razor blade and the underlying striata were scraped into a scintillation vial containing 50pl 1N NaOH. Scintillation fluid (5ml) was added and the vial gently shaken. For the ligand bound to sections, specific binding was determined by subtracting the non-specific binding from total binding. Ligand specifically bound to slices was then calculated in nM knowing the specific activity, the counting efficiency and dilution factors (section 2.8.5).
Figure 2.14. Saturation binding of $^3$H-SCH 23390 to rat striatal slices (A) and Scatchard transformation (B) of the specific saturation data for $^3$H-SCH 23390.

A. Rat forebrain sections (20μm) were incubated for 90 minutes at room temperature in buffer containing $^3$H-SCH 23390 at concentrations ranging from 0.1-4.0nM. Total binding (---), non-specific binding (-----) and specific binding (----) are shown in counts per minute (CPM). Saturation of specific binding occurred at ~2nM, and non specific binding was very low throughout the range of concentrations (<15%).

B. Scatchard transformation of the specific saturation binding data for $^3$H-SCH 23390 (shown in A)

Scatchard transformation revealed a Kd of 0.83nM (obtained from the gradient), and a Bmax (obtained from the y intercept) of 103.9fmols/mg tissue.

Key: B=ligand specifically bound; F=free ligand.
Figure 2.14

A

CPM

8000
6000
4000
2000
0

0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0

3H-SCH 23390 (nM)

B

y = 103.92 - 0.83956x  \quad R^2 = 0.887
**Figure 2.15.** Time course of association of $^3$H-SCH 23390 (specific binding) to rat striatal sections.

Sections were incubated in buffer containing 1nM $^3$H-SCH 23390 for times ranging from 5-150 minutes at room temperature. Association was terminated by rinsing the sections in ice cold buffer. The inset shows a linear pseudo-first order plot of the time course of association, consistent with the reaction obeying simple mass action laws. Equilibrium occurred after about 90 minutes of incubation.
Figure 2.16. Time course of dissociation of $^3$H-SCH 23390 (specific binding) from rat striatal sections.

Sections were first incubated to equilibrium (for 90 minutes) in buffer containing 1nM $^3$H-SCH 23390, and dissociation was initiated by transferring the sections into an 'infinite dilution' of buffer for times ranging from 6-60 minutes. The inset shows a linear first order plot of the time course of dissociation, consistent with the reaction obeying simple mass action laws (Weiland and Molinoff, 1981).
2.8.5. Calculation of results

The following formulae were used to calculate the incubating concentration of ligand in the troughs at the start of experiments (the standard concentrations), the amount of 'free' ligand remaining in the troughs at the end of incubation, and the amount of ligand bound to the striatal sections:

\( \text{STD} \text{ concentration} = \frac{\text{CEM} \times 100 \times \text{dilution factor}}{\text{CE} \times 2.22 \times 10^6 \times \text{sp. act.} (\mu\text{Ci/nmol})} \)

**i. Calculation of standard incubating concentrations:**

**ii. Calculation of free ligand in trough:**

\( \text{FREE ligand} = (\text{Total in STD} \times \text{diln factor}) - \text{Total in sample} \)

\( \text{FREE ligand} = \frac{\text{FREE (CEM)} \times \text{dilution factor} \times 100}{\text{CE} \times 2.22 \times 10^6 \times \text{sp. act.} (\mu\text{Ci/nmol})} \)

**iii. Specific ligand bound to striatal sections**

This was determined by subtracting non-specific from total binding. Initial values obtained in CEM were converted into fmol/mg tissue:

\( \text{Specific binding} = \frac{\text{CEM specific} \times 100}{\text{CE} \times 2.22 \times 10^3 \times \text{sp. act.} \times \text{tissue weight}} \)

For all calculations, \( \frac{100}{\text{CE} \times 2.22} \) converts CPM to Ci, and dividing by specific activity \( (\text{sp. act.} \times 10^3) \) gives final value in fmol.

The mean weight (mg) of 20 representative striatal sections was obtained and the amount of specific ligand bound was calculated in fmol/mg weight tissue.
2.8.6. Graphical analysis of preliminary binding data

For saturation studies, the amount of ligand in the incubation buffer was plotted against the amount of ligand bound in the sections. Scatchard transformation of the specific binding data was used to obtain a value for Bmax and the equilibrium dissociation constant, Kd. Bmax is the maximum number of binding sites for a given ligand; Kd is the equilibrium dissociation constant of the binding reaction.

For kinetic studies, the following analysis was performed according to Weiland and Molinoff (1981) and Bennett and Yamamura (1984):

1. For association of ligand to striatal slices, association time was plotted against ligand bound to sections (CPM) for total, non-specific and specific binding. A pseudo-first order plot of specific binding over the first half life was made. The association rate constant (K+1) was obtained from a pseudo-first order plot.

2. For dissociation, wash time was plotted against ligand bound to striatal slices (CPM) for total, non-specific and specific. A first order plot of wash time versus ln LRT/LRo was obtained, where LRT = ligand bound specifically at time t (mins); and LRo = ligand specifically bound at time 0 mins, i.e. before dissociation was initiated. The dissociation rate constant (K-1) was obtained from the slope of the first order plot.

The equilibrium rate constant, Kd is equal to K-1/K+1.

2.8.7. In vitro slice binding for autoradiography

Brains were prepared as described in section 2.8.1 and 20μm coronal sections through the striatum were mounted in duplicate on slides alternately for total and non-specific binding. Adjacent sections were incubated with ³H-spiperone, ³H-SCH 23390 or ³H-mazindol. Sections for ³H-spiperone (specific activity 29.7Ci/mmol, Amersham) were incubated in TRIS buffer (see section 2.8.2) containing 0.4nM ³H-spiperone (this being approximately equal to the Kd obtained from preliminary binding studies) for 1 hour at room temperature. Non-specific binding was defined with haloperidol 10⁻⁵M and 5HT₂ sites were blocked with ketanserin 10⁻⁵M. Following incubation, sections were rinsed twice in buffer at 4°C for 5 minutes each and then dipped in distilled water for 2 seconds and dried in a stream of cool air.
Sections for \(^{3}\text{H}\)-SCH 23390 binding were incubated for 90 mins at room temperature in TRIS buffer (see section 2.8.3) containing \(^{3}\text{H}\)-SCH 23390 0.2nM (approximately equal to Kd obtained from preliminary kinetic studies, see section 2.8.3). Non-specific binding was defined with CIS-flupenthixol \(10^{-7}\)M. Following incubation sections were rinsed twice consecutively in buffer at 4°C for 5 minutes each, and then dipped for 2 seconds in distilled water. Sections were dried in a stream of cool air.

\(^{3}\text{H}\)-Mazindol binding to striatal sections for autoradiography was performed according to the method of Javitch et al (1984). Binding was performed at 4°C. The assay buffer was TRIS-salts containing 50mM TRIS, 300mM NaCl, 5mM KCl, pH 7.9. Sections for Total binding were incubated for 45 minutes in buffer containing 4nM \(^{3}\text{H}\)-mazindol (Du Pont NEN, UK; Specific activity 15\(\mu\)Ci/mmol), and 0.3m desmethyllumprolamine (DMI) to block noradrenaline uptake sites. Non-specific binding was defined by addition of 10\(\mu\)M mazindol. Incubation was followed by 2 consecutive 1 minute rinses in TRIS buffer, then sections were dipped in distilled water for 2 seconds, and dried in a stream of cool air.

2.8.8. Exposure and development of films

Autoradiograms were generated by apposing slide-mounted tissue sections, and 16 \(^{3}\text{H}\)-microscale reference standards (Amersham; range of tissue-equivalent tritium concentration is 0.06-32nCi/mg wet weight tissue, using 20\(\mu\)m brain sections, Geary et al 1985), to tritium sensitive film (\(^{3}\text{H}\)-Hyperfilm, Amersham) at 4°C for 8 weeks (\(^{3}\text{H}\)-spiperone), 2 weeks (\(^{3}\text{H}\)-SCH 23390) or 6 weeks (\(^{3}\text{H}\)-mazindol). Films were developed in Kodak D19 at 18-22°C, and fixed with Unifix (Kodak) as follows:

1. The film was submerged (emulsion side up to avoid scratching) in a tray containing 2 L of developer, for 5 minutes.
2. The film was then submerged in a tray containing 2 L of tap water for 3 minutes.
3. The film was then submerged in 2 L of fixative (1:4 dilution in tap water) for 8 minutes.
4. The film was washed in a sink of running tap water for 15 minutes.
5. The film was dried on a rack in an upright position.
2.8.9. Computer-assisted densitometry of autoradiograms

The density of binding sites for all 3 ligands were determined using the IBAS Kontron 2000 system. The emulsion response of the film to tritium was calibrated using the Amersham $^3$H-microscales to create a standard curve relating the optical density ('grey value' where 0 = full absorption and 255 = full transmittance) to the known molar quantities of bound ligand (nCi/mg) on the microscales (a typical example is shown in Figure 2.17). The images were corrected for background dishomogeneity. Specific binding was obtained by computerised subtraction of the non-specific image from the total image. The optical density of the area of interest in the autoradiograms was converted into nCi/mg weight tissue from the standard curve, and then to fmols/mg weight tissue by dividing by the specific activity of the ligand. Two to five sections were analysed from each of two or three levels along the rostro-caudal axis of the striatum. These were AP +0.7mm from bregma ('Level 1', rostral to the most anterior placement of foetal grafts or sham grafts), AP +0.2mm, 0.0, and -0.3mm from bregma ('Level 2', through the sites of graft or sham graft placements) and AP -1.0mm to -1.2mm ('Level 3', posterior to the graft or sham graft placements) using the Atlas of Paxinos and Watson (1982). For each level, a mean reading from the 2-5 sections was obtained using a mouse-controlled cursor to define the area of interest. For each section, optical density readings were taken over the whole cross sectional area of the right and left striata ('whole striatum'). For $^3$H-spiperone and $^3$H-SCH 23390, readings were also taken over each of 4 quadrants of the striatum (dorsolateral (DL); ventrolateral (VL); dorsomedial (DM); and ventromedial (VM)) on the right and left for each level (Figure 2.18). Where a graft was visible in any section, it was carefully excluded from the area outlined by the cursor, as ligand binding within the grafts (for all 3 ligands) was consistently low. This was to ensure that any changes in density of binding reflected changes in the surrounding striatum with no contribution from the low levels found within the grafts themselves. Density within the grafts was measured by outlining the grafts with the mouse-controlled cursor. In addition, 'spot' readings were taken over small areas in the immediate vicinity of the grafts.
Figure 2.17. Standard curve for autoradiographic computerised densitometry, using Amersham tritium standards and analysed by the IBAS Kontron system.

An example of a standard curve relating the grey value, obtained in tritium-sensitive film after exposure of Amersham tritium standards, to the known amounts of ligand (nCi/mg) bound in the standards is shown. Due to the greater range in density of $^3$H-SCH 23390 binding to striatal slices as compared with $^3$H-spiperone and $^3$H-mazindol, a larger number of standards was used for preparation of the $^3$H-SCH 23390 standard curve. For all ligands, the amount of ligand bound in the different regions of the striatal sections could then be determined by reading the grey value in the sections from the appropriate standard curve. A new curve was created for each autoradiographic film with tritium standards enclosed in the film cassette together with the striatal sections. Generally a polynomial order 3 was used to join the points on the standard curve.
Figure 2.18. Diagram demonstrating the sub-division of striatum into quadrants for densitometric analysis of autoradiograms.

The striatum was divided arbitrarily into 4 quadrants (dorsolateral, DL; dorsomedial, DM; ventrolateral, VL; and ventromedial, VM.) for each of the 3 AP levels examined. These were Level 1 (1) at AP +0.7mm from bregma; Level 2 (2) at AP +0.2 - -0.3mm from bregma; Level 3 (3) at AP -1.0 - -1.2mm from bregma, using the atlas of Paxinos and Watson (1982).
2.9. STATISTICAL ANALYSIS

For all statistical analyses, a significance level of \( p < 0.05 \) was used.

2.9.1. Apomorphine- and (+)-amphetamine induced behaviour

Mean rotation scores obtained from different tests on the same animals, where the same dose of apomorphine and (+)-amphetamine was used in each case, were compared within each group using a paired Student's t-test. Inter-group comparisons of mean scores were made with ANOVA (where the score prior to graft or sham graft surgery was used as covariate) and post hoc Dunnett's test. Correlations between apomorphine and (+)-amphetamine-induced rotation rates were assessed using Pearson's test. Percentages of animals in each group showing marked apomorphine-induced stereotypy were compared with McNemar's test.

2.9.2. Fluid intake and body weight

Fluid intake and body weight were both compared within each group using a Student's paired t-test. Inter-group comparisons were made with ANOVA and post hoc Dunnett's test.

2.9.3. Histological data

TH-positive cell numbers in the SN or VTA and glial density in the striatum were compared on the right and left sides using a paired Student's t-test. Inter-group comparisons were made with ANOVA and post hoc Dunnett's test. TH-positive cell numbers and glial density within the grafts of animals in the 2 groups were made with an unpaired Student's t-test. Correlations between glial density within the grafts and TH-positive cell numbers were made with a least-squares regression test.

2.9.4. Autoradiographical studies

Autoradiographic density of ligand binding in the right and left striata in each section were compared using Student's t-test. The percentage difference in density of binding on the lesioned/grafted side compared to the intact side was calculated; inter-group comparisons of binding density on the lesioned/grafted striatum were
made with ANOVA, where the binding density on the intact side was used as covariate, and post hoc Dunnett's test.

2.10. EXPERIMENTAL DESIGN

The animals in all the experiments described in this thesis came from 4 large groups of rats which formed the subject of the initial behavioural study (Chapter 3). This study examined the effect of L-DOPA and carbidopa treatment for 5 weeks on motor function (assessed using drug-induced rotation) and stereotypy of rats with a 6-OHDA lesion and sham or foetal grafts. Water intake and body weight were monitored. The experimental groups were as follows:

Group A: 6-OHDA lesion + sham graft (n=22)
Group B: 6-OHDA lesion + sham graft + L-DOPA and carbidopa (n=24)
Group C: 6-OHDA lesion + foetal graft (n=24)
Group D: 6-OHDA lesion + foetal graft + L-DOPA and carbidopa (n=24)

Seven rats chosen randomly from group A were used for HPLC measurement of dopamine in the striata. At the end of all behavioural tests reported in Chapter 3, some rats were selected from each of the 4 groups for \(^3\)H-mazindol autoradiography, to assess dopamine fibre outgrowth, and others were selected for TH-immunohistochemistry to assess dopamine cell survival in the host midbrain and foetal grafts. These investigations are reported in Chapter 4. The density of D-1 and D-2 receptors was also studied using \(^3\)H-sperone and \(^3\)H-SCH 23390 autoradiography in rats used for \(^3\)H-mazindol autoradiography, and this study is described in Chapter 5. Six animals from each of the 4 groups of animals were allocated to a study of the effects of long-term treatment with L-DOPA and carbidopa. This study is described in Chapter 6.

The ways in which rats were used is shown diagramatically in Figure 2.19, and the time-course showing the points at which different procedures were carried out for the purposes of the initial behavioural study, and at which animals were allocated to further studies is shown in Figure 2.20.
Animals were initially divided into 4 large groups (A, B, C and D) which were used for the large behavioural study described in Chapter 3. Subsequently rats from these groups were allocated to further studies as shown in the figure. The results of these further studies are presented in Chapters 4-6.
Figure 2.20. Time course showing the points at which different procedures were carried out, and at which animals were allocated to further studies.

The main events included: lesion and foetal or sham graft surgery; behavioural tests; periods of L-DOPA and carbidopa treatment; allocation of rats to a long-term study of the effects of L-DOPA and carbidopa treatment; and times of killing for immunohistochemical, HPLC, and autoradiographical studies.
Chapter 3

EFFECTS OF TREATMENT WITH L-DOPA AND CARBIDOPA ADMINISTERED IN THE DRINKING WATER ON MOTOR FUNCTION, BODY WEIGHT AND FLUID INTAKE IN 6-OHDA-LESIONED RATS RECEIVING FOETAL VENTRAL MESENCEPHALIC GRAFTS.
3.1. INTRODUCTION

After 2-5 years of treatment of Parkinson's disease with L-DOPA plus a peripheral decarboxylase inhibitor such as carbidopa, the majority of patients develop disabling side effects, and the efficacy of the drug wanes (see section 1.1.7.2). Furthermore, the disease continues to progress. Transplantation of DA-rich tissue, such as that obtained from the human foetal ventral mesencephalon (VM), into the DA-deficient area of the brain may provide an alternative, more physiological means of delivering DA to the striatum. The experimental and clinical use of this technique are outlined in sections 1.3.3 and 1.3.4. The clinical experience in relation to patients receiving foetal transplants has been inconclusive (see section 1.3.4.1). This contrasts with the consistent, reproducible and extensive graft-derived behavioural recovery found in animal models.

One difference between most of the animal studies and the clinical situation is that patients receiving grafts have received concurrent treatment L-DOPA and carbidopa. As outlined in section 1.3.4.2, oxidative metabolism of L-DOPA produces reactive species such as hydrogen peroxide and free radicals, which may damage CA cells by oxidation of sulphydryl groups of proteins, or by initiating lipid peroxidation (Cohen 1986). Quinone by-products of DA metabolism are also potentially cytotoxic (Graham et al 1978). Conceivably, these potentially toxic actions of L-DOPA metabolites could have detrimental effects both on the host DA cells in the substantia nigra and VTA, and also on donor foetal DA cells implanted into the striatum. L-DOPA may also have indirect effects on the function of grafted DA neurons. Thus, L-DOPA (and subsequently dopamine) may inhibit the activity of developing nigral cells and DA released by the grafted cells, by stimulation of presynaptic autoreceptors located on the grafted nerve terminals. However, grafted DA neurons may, if anything,
be less sensitive to inhibition via activation of autoreceptors than intact nigrostriatal cells (Strecker et al 1987). Finally, activation of striatal postsynaptic DA receptors by DA might inhibit the outgrowth of DA fibres (Lankford et al 1988; Lipton and Kater 1989).

These factors might affect the functional recovery produced by foetal VM grafts as well as DA cell survival in the grafts. For these reasons, the effect of chronic treatment with L-DOPA and carbidopa on the functional effects of rat foetal VM grafts in rats has been assessed, using the unilateral 6-OHDA lesioned rotation model of hemi-parkinsonism (Ungerstedt 1971b,c).

3.2. METHODS

3.2.1. Subjects and lesion surgery
Female wistar rats (Bantin and Kingman, Hull, UK) weighing 200-250g at the start of the experiment were used. All animals received a unilateral 6-OHDA lesion of the left medial forebrain bundle, as described in section 2.3.1.

3.2.2. Drug-induced rotation tests
Apomorphine (0.5mg/kg sc)- and (+)-amphetamine (5mg/kg ip)-induced rotation were used to assess the completeness of the lesion and to monitor behavioural effects produced by the graft and by L-DOPA treatment. Apomorphine- and (+)-amphetamine-induced rotation tests were performed, as described in sections 2.4.2 and 2.4.3. Apomorphine-induced stereotypy was also assessed (section 2.4.2.)

3.2.2.1. Assessment of the lesion

Apomorphine induced rotation: One week after lesion surgery rats were tested using apomorphine (0.5mg/kg sc) over a 30 minute period (test 1). Only animals showing a mean of >6 contralateral rotations per minute over the test period were included for further study.
(+) - Amphetamine-induced rotation: Rats were tested with (+)-amphetamine (5mg/kg ip) 1 week after successful testing with apomorphine (test 1). Only rats showing a mean of >8 ipsilateral rotations over 1 hour were included for further study.

Approximately 7-8 weeks after lesion surgery, and immediately prior to foetal or sham graft surgery, the rotational responses to apomorphine (0.5mg/kg sc) and (+)-amphetamine (5mg/kg ip) were reassessed to ensure that there was no reduction in rotational response (test 2).

3.2.2.2. Assessment of the foetal graft and treatment with L-DOPA
Approximately 6 weeks (for (+)-amphetamine) and 8 weeks (for apomorphine) following foetal or sham graft surgery, the rotational responses to both drugs were again assessed (test 3). The rotational rates in response to each drug were compared with those obtained in the respective test performed prior to foetal or sham graft surgery (test 2). The stereotyped response to apomorphine at test 3 was compared with that obtained in test 2.

3.2.3. Biochemical assessment of the lesion
Animals were decapitated and the brain was removed and dissected on an ice-cold surface. Blood for plasma levels was taken from the neck stump. The striatum and limbic areas (olfactory tubercle and nucleus accumbens) were dissected and the lesioned and unlesioned sides were placed separately into individual Eppendorf vials and frozen at -70°C until analysis. DA concentrations were measured in striatum, limbic areas and plasma by high pressure liquid chromatography with electrochemical detection (HPLC-EC), as described in section 2.7.

3.2.4. Experimental groups
Rats (n=94) with approximately equal mean rotation rates in the (+)-amphetamine test performed immediately prior to
foetal or sham graft surgery (test 2) were divided into the following treatment groups:

Group A (n=22): 6-OHDA lesion + sham graft
Group B (n=24): 6-OHDA lesion + sham graft + L-DOPA and carbidopa
Group C (n=24): 6-OHDA lesion + foetal graft
Group D (n=24): 6-OHDA lesion + foetal graft + L-DOPA and carbidopa

Animals were housed in groups of 6 exposed to a 12 hour light:dark cycle, with free access to food. Animals in groups A and C had free access to water, whilst those in groups B and D had free access to water containing L-DOPA and carbidopa (section 3.2.6).

3.2.5. Transplantation surgery
Rat foetal VM cell suspensions were obtained from timed matings of 13-16 days gestation, and prepared as described in section 2.3.2. Cell suspensions were implanted stereotaxically into the 6-OHDA-lesioned striatum (see section 2.3.4). All surgery was completed within 4 hours of preparing the cell suspension, this being optimal for cell viability (Brundin et al 1985a, 1988a). Rats in groups C and D received implants of foetal VM cells into the 6-OHDA lesioned striatum at 3 sites (2μl per site). The same suspension was used to graft equal numbers of rats from groups C and D so as to obtain as much uniformity as possible. Rats in groups A and B received an equivalent volume of glucose saline at the same sites.

In vitro cell viability in the suspensions was assessed at the end of each surgical session (see section 2.3.2.3), and was never below 85%. The mean cell concentration of the suspension was 65,000/μl (including non-viable cells). All surgery was completed in 4 separate surgical sessions performed on consecutive days.
3.2.6. L-DOPA and carbidopa treatment (see section 2.5.)
Drug treatment was commenced 18-24 hours after foetal or sham graft surgery had taken place and continued for 5 weeks. L-DOPA (Roche products, UK) and carbidopa (Merck Sharp and Dohme) were administered in the drinking water at target doses of 200mg/kg/24h and 25mg/kg/24h respectively (see section 2.5). Drug intake was assessed by monitoring the volume of fluid drunk (twice a week) and body weights (weekly) in animals housed in groups of 6. Drug concentration was altered accordingly each week, and fresh solutions were prepared every 3-4 days. Behavioural tests were repeated after L-DOPA and carbidopa treatment had been discontinued for 1 week (see section 3.2.2.2).

3.2.7. Statistical analysis
Within-group changes in fluid intake and body weight were compared using a paired Student's t-test. This test was also used for within-group analysis of rotation rates following administration of apomorphine and (+)-amphetamine in the different tests.

Two-way analysis of variance (ANOVA) was used to compare the differences in mean fluid intake and mean body weight between the 4 groups over the L-DOPA and carbidopa treatment period. This test was also used to compare rotational behaviour between the 4 groups, using the pre-foetal or sham-graft rotational response as covariate, so as to control for any differences that might have existed between the four groups in the first rotation tests. McNemar's test was used to assess within-group changes in apomorphine-induced stereotypy.

3.3. RESULTS
3.3.1. Assessment of the lesion
A total of 120 rats initially received a unilateral 6-OHDA lesion. Ninety-four rats (78%) displayed mean rotation rates of >6 contralateral turns/min in response to apomorphine (0.5mg/kg sc) in test 1. All 94 rats also
showed a mean of >8 ipsilateral turns/minute in response to (+)-amphetamine (5mg/kg ip) in test 1.

Dopamine concentrations in the striata and limbic areas were measured in 7 animals from group A, approximately 20 weeks after lesion surgery. DA concentrations in the intact striatum ranged from 600-1119ng/ml homogenate (mean 838±68.2) but DA was undetectable in the lesioned striatum. For the limbic areas, the mean DA concentration in the intact side was 199.5+ ng/ml, and on the lesioned side was 11.8+ ng/ml. Sample chromatograms are shown in Figure 2.10 (section 2.7).

3.3.2. Fluid intake, body weights and drug treatment

Fluid intake

A 6-OHDA lesion was followed by a fall in fluid intake in all groups within 48 hours, from a mean of 30mls/rat/24h prior to lesion surgery, to means ranging from 19-23 mls/rat/24h (p<0.001, Student's paired t-test) (Figure 3.1). Following a sham graft or foetal graft surgery (groups A and C), fluid intake remained at 19-25 mls/rat/24 hour throughout the rest of the experiment. However, administration of L-DOPA plus carbidopa in the drinking water to animals receiving sham grafts (group B) or foetal grafts (group D) resulted in a further fall in fluid intake in both groups to a mean of 13mls/rat/24h for group B (p<0.001); and a mean of 15.2mls/24h for group D (p<0.01). A 2-way ANOVA for mean fluid intake in the 4 groups over the L-DOPA treatment period revealed a significant effect of drug treatment (F(1,8)=41.3, p<0.0001), with no effect of the foetal graft (F(1,8)=2.8, p=0.13). There was no interaction between the effects of drug treatment and the presence of a foetal graft on fluid intake (F(1,8)=0.12, p=0.48). Fluid intake prior to drug treatment was used as covariate.

After stopping L-DOPA and carbidopa treatment, fluid intake increased rapidly in groups B and D (foetal graft)
Figure 3.1. The mean fluid intake of 6-OHDA-lesioned rats before and after a sham (groups A and B) or foetal graft (groups C and D), with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment for 5 weeks.

Group A - o - ; Group B - • - ; Group C - □ - ; Group D - ■ -

Values shown are mean ± SEM (vertical bars). Horizontal bar represents the period of L-DOPA and carbidopa treatment in groups B and D. Following the 6-OHDA lesion, there was a fall in fluid intake in all groups. There was a further fall in fluid intake in groups B and D at the start of L-DOPA and carbidopa treatment, which persisted for the duration of treatment.

* p < 0.001 compared with pre-lesion intake, Student's t-test.
**p < 0.001 compared to pre-treatment intake in group B, Student's t-test.
+ P < 0.01 compared to pre-treatment intake in group D, Student's t-test.
to levels initially exceeding those found in animals not treated with these drugs (groups A and C). Within 2-3 days after stopping L-DOPA and carbidopa treatment, mean fluid intake in groups B and D had fallen back to levels found in groups A and C.

Body weights
The 6-OHDA lesion was followed by a small fall in body weight in the first week in all groups (not significant), as shown in Figure 3.2. Administration of L-DOPA and carbidopa to animals in group B (sham graft) and group D (foetal graft) produced a further fall in body weight (from a mean of 267±2.7 to a mean of 252±4.7, p<0.01 for group B; and from a mean of 257±4.1 to a mean of 235±3.5, p<0.001 for group D) after 1 week of treatment. The subsequent rates of weight gain were slower in these groups than in rats not receiving L-DOPA and carbidopa (groups A and C). A 2-way ANOVA comparing the mean weights in the 4 groups over the drug treatment period revealed a significant effect of L-DOPA and carbidopa treatment (F(1,7)=38.1, p<0.001), but no effect of foetal graft (F(1,7)=0.56). There was no interaction between the effects of graft and L-DOPA treatment on body weight (F(1,7)=2.53). Mean weights immediately prior to drug treatment were used as covariate (F(1,7)=2.45, p=0.16). Within 24 hours of discontinuing L-DOPA and carbidopa treatment, body weights of animals in groups B and D increased dramatically. Final weights, as measured 2 weeks after drug treatment was discontinued, did not differ in the 4 groups.

Drug treatment
The amount of L-DOPA actually received by the rats ranged from 199-209mg/kg/24h (group B) and 194-255mg/kg/24h (group D); and the amount of carbidopa from 24.8-26.1mg/kg/24h (group B) and 23-31mg/kg/24h (group D).
Figure 3.2. The mean body weight of 6-OHDA-lesioned rats before and after sham graft (groups A and B) or foetal graft surgery (groups C and D), with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment for 5 weeks.

Group A —○— ; Group B —○— ; Group C —□— ; Group D —■—

Values shown are mean ± SEM (vertical bars). The horizontal bar represents the period of L-DOPA and carbidopa treatment in groups B and D. The 6-OHDA lesion was followed by a small (non-significant) fall in body weight in all groups. Commencement of L-DOPA and carbidopa treatment resulted in a fall in body weight by the end of the first week of treatment in groups B and D.

* p<0.01 compared to weight prior to drug treatment of group B, Student's t-test.
** p<0.001 compared to weight prior to drug treatment of group D, Student's t-test.
3.3.3. Motor behaviour

3.3.3.1. Spontaneous behaviour during L-DOPA and carbidopa treatment
During the course of L-DOPA and carbidopa treatment, the 6-OHDA-lesioned rats showed spontaneous rotation away from the lesioned side, which was especially marked in the first 2 weeks of drug treatment. This rotation was often brought about by loud noises or handling. Many rats showed increased grooming, licking and piloerection.

3.3.3.2. (+)-Amphetamine-induced rotation (Figure 3.3)
The mean rotation rates obtained for each group for each (+)-amphetamine test are shown in Figure 3.3A. In all groups there was a small increase in the mean ipsilateral rotation rates between the first and second (+)-amphetamine tests, both of which were performed prior to foetal or sham graft surgery (group A: t=2.77, df=21, p=0.012; group B: t=1.94, df=23, p=0.06; group C: t=2.66, df=23, p=0.014; group D: t=2.11, df=23, p=0.045; Student's t-test). Approximately 6 weeks after sham or foetal graft surgery animals receiving a sham graft only (group A), and those receiving a sham graft followed by 5 weeks' treatment with L-DOPA and carbidopa (group B), continued to show marked ipsilateral rotation in response to the administration of (+)-amphetamine (test 3). In both groups rotation rates increased in successive tests, although the differences between tests 2 and 3 were not significant. The temporal pattern of rotation over the 1 hour test period was unchanged in the 2 groups (Figure 3.3B). Peak ipsilateral rotation occurred approximately 18 minutes after drug injection.

Rats receiving a unilateral 6-OHDA lesion followed by a foetal graft either alone (group C) or followed by L-DOPA and carbidopa treatment (group D) showed complete abolition of ipsilateral rotation following administration of (+)-amphetamine (test 3 compared with test 2: t=8.56,
df=23, p<0.0001 for group C; and t=7.3, df=23, p<0.0001 for group D; Student's t-test). Many animals in both groups displayed mean contralateral rotation over the 1 hour test period (79% in group C and 66% in group D). There was no difference between the 2 groups in the proportion of animals demonstrating 'overcompensation' ($X^2=0.87, p=0.35, \text{chi-squared test}$).

The temporal pattern of rotational response to (+)-amphetamine altered in animals receiving foetal grafts (Figure 3.3B). Following the 6-OHDA lesion, but prior to foetal graft surgery, animals began to rotate ipsilaterally approximately 6 minutes after administration of (+)-amphetamine. Peak rates were obtained at about 18-20 minutes, continuing at a high rate throughout the 1 hour test period. Following foetal graft surgery, most animals began to rotate contralaterally 3-6 minutes after injection of (+)-amphetamine, with peak rates obtained at 6-10 minutes. The rate of contralateral rotation declined fairly rapidly so that by 30-40 minutes after injection most rats had ceased rotating in any direction. This pattern of response was similar in the 2 groups of rats receiving foetal grafts (groups C and D), regardless of treatment with L-DOPA and carbidopa (group D).

The mean rotation rates for the final (+)-amphetamine test (test 3) were compared in all groups using a 2-way ANOVA, with test 2 as covariate. L-DOPA was without effect on behavioural recovery $(F(1,88)=0.068, p=0.5)$. There was a marked effect of the foetal graft on behavioural recovery $(F(1,88)=255.3, p<0.0001)$, with no interaction between the foetal graft and L-DOPA treatment $(F(1,88)=0.069, p=0.79)$.
Figure 3.3A. The mean (+)-amphetamine (5mg/kg ip)-induced rotational rates over a one hour period in 6-OHDA-lesioned rats before and after a sham graft (groups A and B) or foetal graft (groups C and D), with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment.

Group A o—; Group B —; Group C —; Group D —

Values shown are the mean ± SEM (vertical bars). The horizontal bar represents the period of L-DOPA and carbidopa treatment in groups B and D. Test 1 was performed approximately 2 weeks after the 6-OHDA lesion. Test 2 was performed immediately prior to foetal or sham grafting and commencement of L-DOPA and carbidopa treatment. The final test was performed one week after stopping L-DOPA and carbidopa treatment in groups B and D, and 6 weeks after foetal or sham graft surgery. Positive values indicate rotations ipsilateral to the lesioned/grafted side. In all groups there was a tendency for ipsilateral rotation to increase between the first and second tests (p<0.05 for all groups except group B, where the increase was not quite significant: p=0.06, Student's t-test). Rats receiving foetal grafts (groups C and D) demonstrated a reduction in rotation when tested 6 weeks after foetal graft surgery (test 3), as compared to rotation rates prior to grafting (test 2).

*p<0.01 compared with rotational rates immediately prior to foetal graft implantation in groups C and D.

Figure 3.3B. The time course of rotational response to (+)-amphetamine (5mg/kg ip) in 6-OHDA lesioned rats before and 6 weeks after a sham graft (groups A and B) or foetal graft (groups C and D), with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment.

Group A prior to sham graft o—o
Group A 6 weeks after sham graft o—o
Group B prior to sham graft and L-DOPA and carbidopa treatment —
Group B 6 weeks after sham graft and L-DOPA and carbidopa treatment o—o
Group C prior to foetal graft —
Group C 6 weeks after foetal graft —
Group D prior to foetal graft and L-DOPA and carbidopa treatment —
Group D 6 weeks after foetal graft and L-DOPA and carbidopa treatment —

The values shown are the mean ±1SEM number of rotations per minute at 6-10 minute intervals over the 1 hour test period in each group, following administration of (+)-amphetamine 5mg/kg ip. Positive values are rotations ipsilateral to the lesioned/grafted side; negative values are contralateral.
3.3.3.3. Apomorphine-induced rotation and stereotypy
(Figure 3.4 and Table 3.1)
The mean rotation rates obtained in the tests performed after the 6-OHDA lesion but prior to foetal or sham graft surgery (tests 1 and 2) and in that performed 8 weeks later (test 3) are shown in Figure 3.4 for all groups. Contralateral rotation rates increased in all groups (not significant for group A), between the first and second apomorphine tests (group A: $t=1.14$, df=21, $p=0.2$; group B: $t=3.6$, df=23, $p=0.01$; group C: $t=2.64$, df=23, $p=0.015$; group D: $t=3.11$, df=23, $p=0.005$; Student's t-test).

Animals receiving a 6-OHDA lesion followed by a sham graft alone (group A) continued to show marked contralateral rotation in the final apomorphine test (test 3 compared with test 2; $t=0.47$, df=21, $p=0.64$; Student's t-test). There was a small, non-significant increase in the proportion of animals showing marked stereotypy ($p=0.2$, Table 3.1). Rats receiving a unilateral 6-OHDA lesion followed by a sham graft and 5 weeks' treatment with L-DOPA and carbidopa (group B) showed a further small increase in contralateral rotation (test 3 compared with test 2; $t=2.16$, df=20, $p=0.053$). In group B there was also an increase in the proportion of animals showing stereotypy ($p<0.05$, McNemar's test). Animals receiving a unilateral lesion followed by a foetal graft (group C) showed a decrease in apomorphine-induced contralateral rotation compared with the rates observed in the test performed immediately prior to foetal graft surgery (test 3 compared with test 2: $t=2.76$, df=21, $p=0.012$). There was a small decrease in the proportion of rats showing marked stereotypy (not significant). Rats receiving a unilateral lesion followed by a foetal graft and 5 weeks' treatment with L-DOPA and carbidopa (group D) showed a reduction in mean rotation rates (test 3 compared with test 2: $t=3.94$, df=23, $p=0.001$). There was a small non-significant increase in the proportion of animals showing marked stereotypy ($p=0.8$; McNemar's test).
Figure 3.4. The mean rotation rates over 30 minutes immediately following administration of apomorphine (0.5mg/kg sc) in 6-OHDA lesioned rats before and after a sham graft (groups A and B) or foetal graft (groups C and D), with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment.

Group A — ; Group B — ; Group C □ — ; Group D ■ —

Values shown are mean ± SEM (vertical bars). The horizontal bar represents the period of treatment with L-DOPA and carbidopa for groups B and D. The first test was performed approximately 1 week after the 6-OHDA lesion. Test 2 was performed immediately prior to sham or foetal graft surgery. The final test was performed 3 weeks after stopping L-DOPA and carbidopa in groups B and D, and 8 weeks after foetal or sham graft surgery. Contralateral rotation increased for all groups except group A between the first and second tests. Rotation was subsequently unchanged in animals from groups A and B (test 3 compared with test 2). Groups C and D showed a reduction in contralateral rotation between the final test and that immediately preceding foetal graft surgery.

* p<0.05 and ** p<0.01 compared to rotation prior to foetal graft surgery (groups C and D respectively), Student's t-test.
Table 3.1. The proportion of 6-OHDA lesioned rats showing stereotypy following administration of apomorphine (0.5mg/kg sc) before and after a sham graft (groups A and B) or foetal graft (groups C and D), with (groups B and D) or without (groups A and C) L-DOPA and carbidopa treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-foetal/sham graft</th>
<th>Post-foetal/sham graft</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7/24</td>
<td>12/23</td>
</tr>
<tr>
<td>B</td>
<td>5/24</td>
<td>11/24*</td>
</tr>
<tr>
<td>C</td>
<td>7/24</td>
<td>4/23</td>
</tr>
<tr>
<td>D</td>
<td>4/24</td>
<td>9/23</td>
</tr>
</tbody>
</table>

* p<0.05 when the post-sham graft score is compared with pre-sham graft score, McNemar's test.

All animals received a unilateral 6-OHDA lesion prior to the first test. Subsequently rats in groups A and B received sham grafts and groups C and D received foetal dopamine grafts. Groups B and D received L-DOPA and carbidopa for 5 weeks in the drinking water before all rats were retested. The first column refers to the test performed just prior to foetal or sham graft surgery and commencement of L-DOPA treatment. The second column refers to the test performed 8 weeks after foetal or sham graft surgery (3 weeks after stopping L-DOPA and carbidopa in groups B and D).
The apomorphine-induced rotation rates obtained in test 3 were compared in all 4 groups using a 2-way ANOVA, with test 2 as covariate. There was no significant effect of L-DOPA and carbidopa treatment on behavioural recovery (F(1, 84) = 1.64, p = 0.2). There was a significant effect of foetal graft on the behavioural recovery (F(1, 84) = 10.665, p < 0.05), with no interaction between the foetal graft and L-DOPA treatment (F(1, 84) = 1.323, p = 0.2).

3.4. DISCUSSION

These results indicate that administration of L-DOPA and carbidopa for 5 weeks in the drinking water to rats receiving rat foetal VM grafts does not inhibit the functional effects of the grafts as revealed by the reduction of drug-induced rotation. Several points have been demonstrated:

(1) Robustness of the 6-OHDA lesion was demonstrated by drug-induced rotational behaviour and striatal DA concentrations.
A unilateral 6-OHDA lesion of the nigrostriatal pathway resulted in apomorphine-induced contralateral rotation consistent with >98% DA denervation of the striatum on one side (Ungerstedt 1971c; Hefti et al 1980). The completeness of the lesion was confirmed by marked ipsilateral rotation to (+)-amphetamine (Schmidt et al 1983). The lesion-induced motor asymmetry was not altered by the presence of a sham graft, as the rotational responses to both apomorphine and (+)-amphetamine persisted through to the final rotation tests. This demonstrates both the robustness of the lesion and the lack of effect of sham graft on behavioural recovery.

DA concentrations were reduced so severely in the 6-OHDA lesioned striatum as to be undetectable by the HPLC-EC assay system. DA depletion in the limbic areas ipsilateral to the 6-OHDA lesion was less marked. This
supports the behavioural data which suggested that the lesioned animals had >98% depletion of DA in the lesioned striatum, consistent with complete loss of DA cells in the ipsilateral substantia nigra, but a less severe DA cell loss in the VTA.

(2) Foetal grafts reduced or abolished drug-induced motor asymmetry, and L-DOPA treatment did not alter this effect.

In contrast to the persistent rotational responses to apomorphine and (+)-amphetamine shown by rats receiving a unilateral lesion followed by a sham graft (group A), or a sham graft and L-DOPA treatment (group B), animals receiving a foetal graft alone (group C) or with L-DOPA treatment (group D) showed a reduction of apomorphine-induced contralateral rotation and abolition of (+)-amphetamine-induced ipsilateral rotation. For both tests, there was no difference in the extent of recovery in groups C and D. This indicates that L-DOPA treatment for 5 weeks did not interfere with the functional effects of foetal VM grafts as studied with this model. Furthermore, the proportion of rats showing a net mean contralateral rotation ('overcompensation') following administration of (+)-amphetamine after receiving a foetal graft did not differ in the 2 groups. Contralateral rotation occurred earlier in the test period following foetal graft implantation than did ipsilateral turning prior to grafting, and was sustained for a shorter time. The exact mechanism underlying overcompensation remains unknown, but it may involve a hyper-reactivity of the grafted neurones to (+)-amphetamine (Herman et al 1985). L-DOPA treatment did not alter the time course of rotation following foetal grafting, which may suggest that L-DOPA did not interfere with the turnover, storage or release of DA by the transplanted nerve terminals.

The reduction of apomorphine-induced contralateral rotation in animals receiving foetal grafts (groups C and
D) was small. This may have been due to the high dose used (0.5mg/kg sc) and to the relatively short period between graft surgery and the final apomorphine test (8 weeks). Nevertheless, it could be indicative of spontaneous DA release by the grafted cells. In this study, treatment of animals which had received a unilateral lesion and a sham graft with L-DOPA tended to result in a further increase in apomorphine-induced contralateral rotation above that due to the lesion alone (compare rotations in group B with group A in test 3, Figure 3.4). In contrast, the presence of a foetal graft in animals treated chronically with L-DOPA (group D) not only reduced the behavioural supersensitivity resulting from the 6-OHDA lesion, but also prevented further supersensitivity developing as a result of the L-DOPA treatment. Similarly, Gaudin et al (1990), using a different L-DOPA treatment regime from the present experiment, noted that foetal DA cell grafts prevented the development of behavioural supersensitivity associated with chronic exposure to L-DOPA.

(3) L-DOPA treatment of 6-OHDA lesioned rats did not reduce drug-induced rotation, but did result in an increase in apomorphine-induced stereotypy (AIS).

Rotational responses to apomorphine and (+)-amphetamine persisted in rats receiving sham grafts and L-DOPA treatment (group B), which demonstrates that L-DOPA treatment for a period of 5 weeks did not, on its own, reduce rotation. Indeed, there was a small increase in apomorphine-induced contralateral rotation in animals in group B, which was not quite significant. Excessive stereotypy may have limited the increase in circling rates in this group.

A 6-OHDA lesion followed by a sham graft (group A) was associated with a small, non-significant increase in AIS over time, whereas animals receiving a unilateral 6-OHDA
lesion followed by a foetal graft (group C) showed a small decrease. Unilateral 6-OHDA lesions have previously been reported to reduce (Loew et al 1975) or enhance (Costall et al 1977) stereotyped behaviour. Administration of L-DOPA to animals with a unilateral 6-OHDA lesion and a sham graft (group B) resulted in a marked increase in AIS. Hall et al (1984) noted an increase in stereotypy in normal rats treated chronically with L-DOPA. In the present study, animals receiving a 6-OHDA lesion followed by a foetal graft and chronic treatment with L-DOPA (group D) did not show a significant increase in AIS, suggesting that the foetal graft helped to prevent exacerbation of behavioural supersensitivity by L-DOPA treatment.

(4) A 6-OHDA lesion and chronic L-DOPA treatment resulted in a fall in body weight and fluid intake.

A unilateral 6-OHDA lesion was associated with weight loss and hypodipsia in all animals. Severe adipsia and aphagia have been reported previously in rats with bilateral 6-OHDA lesions of the nigrostriatal pathway (Ungerstedt 1971d; Dunnett et al 1981b). The less extreme weight loss and hypodipsia seen in this experiment may reflect a less severe DA deficiency than in the bilateral-lesion syndrome. The foetal grafts implanted into these adult rats had no effect on fluid intake or body weight. Rogers et al (1990) reported that foetal DA grafts implanted unilaterally into the posterior-ventral striatum of neonatal animals partially protected against the severe adipsia and aphagia that follows adult bilateral 6-OHDA lesions. This contrasts with the failure of such grafts to afford similar protection or recovery from 6-OHDA lesions when they are implanted into adult rats (Dunnett et al 1981b).

Administration of L-DOPA and carbidopa produced a further fall in fluid intake and body weight, but not severe enough to warrant tube feeding. For the duration of L-
DOPA treatment, fluid intake remained low and the rate of weight gain was substantially less than in the untreated animals. Part of this weight loss may be due to the reduction of fluid intake as, within days of stopping drug treatment, fluid intake and body weight recovered simultaneously to levels found in the untreated groups. Whether a reduction in food intake contributed to the weight loss is not known. Increased energy expenditure due to the increase in spontaneous circling rates and locomotor activity in L-DOPA treated animals may also contribute to the weight loss (Pycock et al 1982; Karoum et al 1988).

The question of L-DOPA toxicity

In this experiment, treatment with L-DOPA and carbidopa for 5 weeks had no detrimental effects on the functional recovery produced by foetal VM grafts in this rat model of Parkinson's disease. The plasma L-DOPA levels produced by this regime (see section 2.5) were similar to those found clinically (Hare et al 1973). However, toxic effects of L-DOPA on the grafted DA neurones could still be occurring which are not detectable with the behavioural tests used in this study. Thus, complete abolition of (+)-amphetamine-induced rotation can occur with 300-500 surviving DA cells in the implanted striatum (Brundin et al 1985a, 1988a). It is therefore possible that L-DOPA could have reduced survival of cells in the grafts to an extent which was not sufficient to affect the behavioural recovery. For this reason, some animals from the large groups used in this study were examined for DA cell survival and fibre outgrowth using tyrosine hydroxylase immunohistochemistry and 3H-mazindol autoradiography respectively (Chapter 4). Finally, L-DOPA and carbidopa were administered in this study only for 5 weeks. Longer periods might prove detrimental to graft function and survival. This question is addressed in Chapter 6.
4.1. INTRODUCTION

Chapter 3 described how foetal ventral mesencephalic (VM) grafts implanted into the 6-OHDA lesioned striatum resulted in abolition of (+)-amphetamine-induced ipsilateral rotation and reduction of apomorphine-induced contralateral rotation. These effects were not altered by treatment with L-DOPA and carbidopa in the drinking water for 5 weeks. Furthermore, the foetal grafts prevented the development of marked apomorphine-induced stereotypy that was otherwise observed in 6-OHDA lesioned, sham-grafted rats treated chronically with L-DOPA and carbidopa.

Reduction of (+)-amphetamine-induced ipsilateral rotation has been shown to be a better index of dopamine (DA) cell survival in the grafts than reduction in apomorphine-induced contralateral rotation (Dunnett et al 1988). Even so, the survival of DA cells within these grafts, and the integrity of their fibre outgrowth, cannot be directly inferred from behavioural effects of the grafts alone. Thus, in the 6-OHDA rat model, reduction of (+)-amphetamine-induced ipsilateral rotation is dependent on the number of surviving DA cells within the grafts; but when abolition of ipsilateral rotation has been achieved, there is no way of knowing whether L-DOPA has nevertheless had some detrimental effect on graft survival. Using solid tissue implants, Bjorklund et al (1980b) found an excellent correlation between the extent of DA fibre outgrowth into the denervated striatum and reduction of (+)-amphetamine-induced rotation in 6-OHDA lesioned rats, up to a point: complete recovery from rotational asymmetry was obtained with reinnervation of one-eighth of the head of the striatum. Similarly, using cell suspensions, Brundin et al (1985a, 1988a) showed a correlation between the number of surviving DA cells and the percentage reduction of (+)-amphetamine-induced rotation up to the survival of 300-500 DA cells, beyond which point total behavioural recovery occurred.
So, it is possible that the L-DOPA treatment may have had a detrimental effect on survival of DA cells in the grafts, or on their morphology, which was not sufficiently great to affect the behavioural recovery reported in Chapter 3. For these reasons, the survival of DA cells and their fibre outgrowth were assessed in animals selected on the basis of having shown 80-100% reduction of (+)-amphetamine-induced ipsilateral rotation following grafting. DA cell survival was assessed with tyrosine hydroxylase immunohistochemistry (TH-IHC), and DA nerve terminal density was assessed using ³H-mazindol autoradiography as an index of fibre outgrowth (Javitch et al 1984).

4.2. METHODS

4.2.1. Animal subjects
Animals from the groups studied in the large behavioural experiment were used (see section 3.2.4). Representative animals from each of the 4 groups were studied with either TH-IHC or ³H-mazindol autoradiography, as follows:

Animals for TH-IHC:
- **Group A**: 6-OHDA lesion + sham graft (n=3)
- **Group B**: 6-OHDA lesion + sham graft + L-DOPA and carbidopa (n=3)
- **Group C**: 6-OHDA lesion + foetal graft (n=6)
- **Group D**: 6-OHDA lesion + foetal graft + L-DOPA and carbidopa (n=6)

Animals for ³H-mazindol autoradiography:
- **Group A**: 6-OHDA lesion + sham graft (n=4)
- **Group B**: 6-OHDA lesion + sham graft + L-DOPA and carbidopa (n=3)
- **Group C**: 6-OHDA lesion + foetal graft (n=5)
- **Group D**: 6-OHDA lesion + foetal graft + L-DOPA and carbidopa (n=6)
Animals in the sham-grafted groups (A and B) were selected from the larger groups of rats studied in Chapter 3, on the basis of showing persistent high rates of ipsilateral rotation to (+)-amphetamine and contralateral rotation to apomorphine in all tests (see section 3.3.3). All rats in the foetal-grafted groups (C and D) used for TH-IHC had shown >100% reduction of (+)-amphetamine-induced ipsilateral rotation in test 3 (see section 3.3.3.2). Those animals in the foetal grafted groups used for 3H-mazindol autoradiography had shown at least 80% reduction of (+)-amphetamine-induced ipsilateral rotation in test 3, and 40-50% reduction in apomorphine-induced contralateral rotation in test 3. Animals for TH-IHC were killed approximately 10 weeks after foetal or sham graft surgery (i.e. within 2 weeks of the final apomorphine test: see Figure 2.20 and section 3.3.3.3). Animals for autoradiography were killed approximately 8.5 weeks after foetal or sham graft surgery (i.e. within 2 days of the final apomorphine test). By the time of killing, animals in groups B and D had received treatment with L-DOPA and carbidopa for 5 weeks at target doses of 200mg/kg/24h and 25mg/kg/24h respectively.

4.2.2. Tyrosine hydroxylase immunohistochemistry (TH-IHC)
Animals were anaesthetised with sodium pentobarbital (50mg/kg ip) and perfused via the left ventricle with 4% paraformaldehyde in phosphate buffered saline (PBS) as described in section 2.6.1. Coronal sections (40µm thick) were cut on a vibratome through the entire striatum and substantia nigra into PBS. Immunostaining for TH was performed as described in section 2.6.4).

4.2.3. Cell counting and graft volume estimation
For the substantia nigra (SN) and ventral tegmental area (VTA), TH-positive cells were counted separately on the intact and lesioned sides according to the method of Konigsmark (1970) (see section 2.6.5.1). Three sections taken at AP -5.8 from bregma, using the atlas of Paxinos
and Watson (1982), were selected from each of the grafted animals and mean cell numbers determined. No attempt was made to estimate the total number of cells throughout these nuclei.

TH-positive cell numbers within the graft and graft volume were assessed, as described in section 2.6.5.1. Estimation of fibre outgrowth from the grafts was made by measuring the distance between the edge of the cell bodies in the grafts and the point at which TH-positive fibres emerging from the grafts ceased.

4.2.4. Statistical analysis of histological data
For the SN and VTA of animals in groups C and D (receiving foetal grafts), cell counts on the intact and lesioned side were compared using a paired Student's t-test. Cell numbers in the intact SN of group C (lesion followed by foetal graft alone) were compared with those found in group D (lesion followed by foetal graft and treatment with L-DOPA and carbidopa) using Student's independent t-test. Total cell numbers within the grafts and graft volumes found in groups C and D were also compared using Student's independent t-test. Pearson's test was used to assess the relationship between cell numbers in the grafts and graft volumes.

4.2.5. 3H-Mazindol autoradiography
Animals were anaesthetised with pentobarbital 50 mg/kg ip and perfused via the left ventricle with 100 ml of 5% dextrose saline 8 weeks after foetal graft or sham graft surgery (i.e. 3 weeks after discontinuation of L-DOPA and carbidopa treatment in groups B and D). Brains were prepared as described in section 2.8.1. Coronal sections (20μm thick) were cut through the striatum on a Bright Instruments cryostat at -20°C, and stored at -20°C until binding studies were carried out.

3H-Mazindol (DuPont NEN, U.K.; specific activity 15
Ci/mmol) binding was performed according to the method of Javitch et al (1984, 1985b), as described in section 2.8.7. Autoradiograms were generated by apposing slides to tritium Hyperfilm (Amersham) for 6 weeks at 4°C (Unnerstall et al 1982). Included in each cassette were 16 Amersham tritium microscale reference standards (range of tissue-equivalent tritium concentration 0.06-32.0 nCi/mg tissue wet weight, [Geary et al 1985]). Films were developed in Kodak D19 (see section 2.8.8).

4.2.6. Computer-assisted densitometry
Using computerised densitometry (IBAS-2000; Kontron) to analyse the autoradiograms, the density of the binding sites was used to quantify the dopaminergic innervation of the striatum as derived from the host nigrostriatal pathway or from foetal nigral cells implanted into the denervated striatum. Details of the procedure are described in section 2.8.9. In brief, the emulsion response of the film to tritium was calibrated by using Amersham tritium microscales to create a standard curve relating the optical density to the known molar quantities of bound ligand (nCi/mg) on the microscales. The image was corrected for background dishomogeneity. Specific binding was obtained by computerised subtraction of the non-specific image from the total image.

Four or five coronal sections taken at two anterior-posterior (AP) levels through the striatum in each animal were selected for quantification of 3H-mazindol binding. The most anterior section "Level 1" was rostral to the most anterior placement of foetal DA or sham graft, and corresponded to AP +0.7 from bregma (Paxinos and Watson 1982). The subsequent sections were taken through one or more of the foetal/sham graft placements ("Level 2") and corresponded to AP +0.2, 0.0, and -0.3 from bregma. For each section, readings were taken over the whole cross-sectional area of the striatum on each of the intact and lesioned/grafted sides. "Spot" readings were also taken
directly over areas assumed to be graft-derived binding sites.

4.2.7. Statistical analysis of autoradiograms
Level 1 and Level 2 were analysed separately, and for Level 2 mean values were calculated from the 3-4 sections for each of the intact and lesion/grafted sides for each animal. Within each group, the difference in ligand binding on the intact side and the lesioned/grafted side was compared using a paired Student's t-test. Two-way ANOVA was used to compare the density of ligand binding on the intact side and on the lesioned/grafted side in animals from all 4 groups, for Level 1 and 2. Square-root transformation of the raw data was also used to reduce the effects of variation in level and spread.

4.3. RESULTS

4.3.1. Behavioural assessment
The apomorphine-induced rotational responses of the animals used in this study are shown in Table 4.1 (animals used for TH-IHC in part A, and animals for autoradiography in part B); and their (+)-amphetamine-induced rotational responses are shown in Table 4.2 (animals used for TH-IHC in part A, and animals for autoradiography in part B). The same pattern of drug-induced rotation was seen for the different groups as those described for the larger groups from which they were taken in Chapter 3. In brief, rats in groups A and B continued to show marked contralateral rotation following administration of apomorphine (0.5mg/kg sc), when tested 8 weeks after sham graft surgery (Table 4.1). These animals also continued to show marked ipsilateral rotation following administration of (+)-amphetamine (5mg/kg ip) when tested 6 weeks after sham graft surgery (Table 4.2). In contrast, rats in groups C and D demonstrated 40-50% reduction in apomorphine-induced contralateral rotation when tested 8 weeks after foetal graft surgery (Table 4.1); and 80-100% reduction of (+)-
Table 4.1. Rotational rates of 6-OHDA lesioned rats following administration of apomorphine (0.5mg/kg sc) before and after sham graft (groups A and B) or foetal graft (groups C and D) either alone (groups A and C) or following treatment with L-DOPA and carbidopa (Groups B and D) for those animals subsequently examined using TH immuno-histochemistry (A) or ^3H-mazindol binding to striatal slices (B).

<table>
<thead>
<tr>
<th>(A)</th>
<th>Mean rotational rates</th>
<th>Post-SG/FG (test 3)</th>
<th>Pre-SG/FG (test 2)</th>
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<tbody>
<tr>
<td>Group A (n = 3)</td>
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<td>Group B (n = 3)</td>
<td>11.6±3.1</td>
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<td>Group C (n = 6)</td>
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<td>Group D (n = 6)</td>
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<table>
<thead>
<tr>
<th>(B)</th>
<th>Mean rotational rates</th>
<th>Post-SG/FG (test 3)</th>
<th>Pre-SG/FG (test 2)</th>
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<td>Group A (n = 4)</td>
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<tr>
<td>Group B (n = 3)</td>
<td>12.5±1.7</td>
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<tr>
<td>Group C (n = 5)</td>
<td>12.1±1.8</td>
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<tr>
<td>Group D (n = 6)</td>
<td>13.8±3.4</td>
<td><strong>6.2±2.0</strong></td>
<td></td>
</tr>
</tbody>
</table>

** P<0.01, and * P<0.05 compared to pre-foetal graft score.

The net mean (+ 1 SEM) rotational scores per minute obtained over 30 minutes immediately following administration of apomorphine (0.5 mg/kg sc) are shown. All animals had received a unilateral 6-OHDA lesion. Rats in groups A and B received sham grafts (SG), whilst groups C and D received foetal grafts (FG) into the denervated striatum. Groups B and D received 5 weeks treatment with L-DOPA and carbidopa immediately after SG/FG surgery. Tests were performed immediately prior to sham or foetal graft surgery (pre SG/FG, test 2 in Chapter 3) and 8 weeks later (post SG/FG, test 3 in Chapter 3). Positive values are contralateral turns.
Table 4.2. Rotational rates of 6-OHDA lesioned rats following administration of (+)-amphetamine before and after sham graft (groups A and B) or foetal graft (groups C and D) either alone (Groups A and C) or following treatment with L-DOPA and carbidopa (groups B and D) for those animals subsequently examined using TH immunohistochemistry (A) or $^3$H-mazindol binding to striatal slices (B).

<table>
<thead>
<tr>
<th></th>
<th>Mean rotational rates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-SG/FG (test 2)</td>
</tr>
<tr>
<td></td>
<td>Post-SG/FG (test 3)</td>
</tr>
<tr>
<td><strong>(A)</strong></td>
<td></td>
</tr>
<tr>
<td>Group A (n = 3)</td>
<td>12.6±2.2</td>
</tr>
<tr>
<td></td>
<td>13.5±2.3</td>
</tr>
<tr>
<td>Group B (n = 3)</td>
<td>14.6±2.4</td>
</tr>
<tr>
<td></td>
<td>15.2±3.1</td>
</tr>
<tr>
<td>Group C (n = 6)</td>
<td>13.9±2.1</td>
</tr>
<tr>
<td></td>
<td>-3.4±0.5*</td>
</tr>
<tr>
<td>Group D (n = 6)</td>
<td>15.4±2.8</td>
</tr>
<tr>
<td></td>
<td>-3.0±1.3*</td>
</tr>
<tr>
<td><strong>(B)</strong></td>
<td></td>
</tr>
<tr>
<td>Group A (n = 4)</td>
<td>15.0±2.3</td>
</tr>
<tr>
<td></td>
<td>14.8±0.6</td>
</tr>
<tr>
<td>Group B (n = 3)</td>
<td>15.7±6.0</td>
</tr>
<tr>
<td></td>
<td>17.6±4.4</td>
</tr>
<tr>
<td>Group C (n = 5)</td>
<td>13.7±2.2</td>
</tr>
<tr>
<td></td>
<td>-1.0±1.8*</td>
</tr>
<tr>
<td>Group D (n = 6)</td>
<td>18.1±5.4</td>
</tr>
<tr>
<td></td>
<td>-2.3±0.5*</td>
</tr>
</tbody>
</table>

* P<0.01 compared to pre-foetal graft score.

The net mean rotational scores (+ 1 SEM) per minute obtained over 1 hour immediately following administration of (+)-amphetamine (5 mg/kg ip) are shown. All animals had received a unilateral 6-OHDA lesion. Groups A and B received sham grafts (SG), whilst groups C and D received foetal grafts (FG) into the denervated striatum. Groups B and D received 5 weeks treatment with L-DOPA and carbidopa immediately after SG/FG surgery. Tests were performed immediately prior to sham or foetal graft surgery (pre FG/SG; test 2 in Chapter 3) and 6 weeks later (post FG/SG; test 3 in Chapter 3). Positive values are ipsilateral turns, negative values are contralateral.
amphetamine-induced ipsilateral rotation when tested 6 weeks after foetal graft surgery, with some rats in each group rotating showing a net mean contralateral rotation (Table 4.2).

4.3.2. TH immunohistochemical study of the midbrain and striatum

Gross morphology
Macroscopically, the lesioned SN appeared shrunken compared with the intact SN in all animals studied. There was no difference in the gross appearance of the SN and VTA on either side in rats that had received treatment with L-DOPA and carbidopa (groups B and D) and those that had not (groups A and C). Examination of the forebrain revealed an enlarged lateral ventricle on the side ipsilateral to the lesion, with shrinkage of the adjacent striatum.

TH Immunohistochemistry
(a) SN and VTA (Table 4.3):
The ventral mesencephalon at AP -5.8 from bregma (Paxinos and Watson, 1982) was studied in animals from both groups receiving foetal DA cell grafts (groups C and D). On the non-lesioned side, TH-positive cell diameters in both the VTA and SN ranged from 8 to 20 μm. The larger cells were fusiform in shape. There was no difference between the groups in the number of TH-positive cells in the intact SN and VTA at this level. The unilateral 6-OHDA lesion produced a virtual disappearance of TH-positive cells in the lesioned SN to <3% of the intact side (p<0.001 for both groups). The extent of cell loss in the 6-OHDA-lesioned VTA was more variable than that in the lesioned SN (0-66% of the intact side), but still significantly reduced (p<0.001 both groups). The number of TH-positive cells remaining in the 6-OHDA-lesioned VTA was further reduced by L-DOPA and carbidopa treatment for 5 weeks (F(1,11)=6.6, p=0.03, ANOVA, with the intact side as covariate; post hoc Dunnett's test was not significant).
Table 4.3. The mean number of TH-positive cells in the SN and VTA in sections taken through the ventral mesencephalon of animals with a unilateral 6-OHDA lesion receiving foetal grafts alone (group C) or foetal grafts followed by L-DOPA and carbidopa treatment (group D).

<table>
<thead>
<tr>
<th>Group</th>
<th>Intact side</th>
<th>Lesioned side</th>
<th>% Intact</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SN</td>
<td>VTA</td>
<td>SN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group C</td>
<td>183</td>
<td>88</td>
<td>1.4*</td>
</tr>
<tr>
<td>(n=6)</td>
<td>+20.5</td>
<td>+9.7</td>
<td>+0.8</td>
</tr>
<tr>
<td>Group D</td>
<td>166</td>
<td>104</td>
<td>0.9*</td>
</tr>
<tr>
<td>(n=6)</td>
<td>+14.8</td>
<td>+15.1</td>
<td>+0.6</td>
</tr>
</tbody>
</table>

* P<0.001 compared with intact side.

Values shown are mean ± 1 SEM. The number of cells on the lesioned side is expressed as a percentage of the intact side, with the range shown in brackets. All animals had received a unilateral 6-OHDA lesion of the nigro-striatal pathway followed by a foetal DA graft into the denervated striatum. Animals in group D received 5 weeks treatment with L-DOPA and carbidopa following foetal cell implantation.
(b) Intra-striatal ventral mesencephalic grafts:
No TH-immunoreactive fibres remained in the lesioned striatum of rats receiving sham grafts, and no TH-positive cells were identified. In rats receiving intrastriatal nigral grafts with (group D) or without (group C) L-DOPA and carbidopa treatment, TH-positive neurones were identified in the grafted striata of all animals examined. Grafts were found dorsally in the anterior striatum, and laterally in the mid striatum (Figure 4.1A). Some grafts were located just beneath the corpus callosum. In the most caudal sections, some grafts were located ventrally extending into the globus pallidus. Two grafts were occasionally found in the same coronal section. In most cases, however, the two grafts which had been placed one above the other in the lateral striatum had merged to form a single mass, whereas the graft placed more anteriorly was usually clearly distinguished.

The distribution of the cells within the grafts was similar in groups C and D. Thus, TH-positive cells were commonly found in clusters around the periphery of the graft, or at its dorsal or ventral poles (Figure 4.1A). The central area of the grafts was relatively cell-free, although occasional strands of cells could be seen running across it. This pattern was most obvious in grafts of large cross-sectional area. Two types of cell were found in the grafts: a small rounded cell of 10-12 μm in diameter, and a larger spindle shaped cell of ~20 μm. Macrophages were found in portions of some grafts.

A halo of TH-positive fibres could be seen extending from the grafts for distances of up to 1mm into the surrounding striatum. The density of TH-positive reinnervation was not quantified, but in areas adjacent to the graft appeared to be as high as that found in the contralateral striatum. Fine TH-positive fibres emanating from cells in grafts from two animals treated
with L-DOPA and carbidopa are shown in Figures 4.1B and 4.2. In addition, cells were identified with large branching processes, probably dendrites (Figure 4.3). The central area of the grafts was relatively free of fibres, although dense strands of TH-positive processes could sometimes be seen running from one aggregate of cells across to the other side. Grafts located just below the corpus callosum showed no evidence of fibre extension into the overlying areas.

The number of TH-positive cells in each graft was counted. Where two grafts were separately identifiable in any one animal, the cell numbers were combined to give a single value. There was no difference in the total number of TH-positive cells found in grafts of animals receiving L-DOPA and carbidopa (group D) and those receiving grafts alone (group C): in group D mean cell number = 1604±465 SEM, range 226-3335; and in group C mean cell number = 1392±338 SEM, range 123-2918 (Student's t-test: t=0.35, df=10, p=0.73).

In the estimation of graft volumes, where two or more grafts were separately identifiable in any animal, the volumes were combined to give a single value. The total graft volumes in animals treated with L-DOPA and carbidopa (group D) ranged from 0.6 to 3.5 mm³ (mean 1.8±0.48 SEM) and in animals receiving grafts alone (group C) from 0.65 to 3.7 mm³ (mean 1.4±0.5 SEM), with no difference between the groups (Student's t-test: t=0.62, df=10, p=0.55).

As there was no difference between the two groups with respect to either total cell numbers in the grafts or graft volumes, the data were combined to assess the relationship between graft volume and cell number. Pearson's test showed a positive correlation between graft volume and graft cell number in these animals (r=0.85, p<0.01).
Figure 4.1. Photomicrographs of TH immunostained cells located in a foetal VM implant in an animal treated with L-DOPA and carbidopa.

A: Low power photomicrograph (immunostained for TH) of part of the dorsal striatum showing a foetal graft taken from a 6-OHDA lesioned animal that had received treatment with L-DOPA and carbidopa for 5 weeks. Scale bar=120μm. TH-positive cells are clustered at the dorsal pole and periphery of the graft with a relatively cell-free centre. The corpus callosum is just visible at the top left corner. The arrowhead marks cells with fine long fibres at the ventro-medial edge of the graft (shown at higher magnification in Fig. 4.1B).
Figure 4.1B. Higher magnification of TH-positive cells and fibres (single arrowhead in Fig. 4.1A) located at the ventral pole of the graft in A. TH-immunoreactive processes (single arrowheads) are seen running across the width of the graft to reach the host striatum on the other side. Scale bar=20μm.
Figure 4.2. High power view of a fine calibre TH-positive fibre taken from a graft of an animal treated with L-DOPA and carbidopa (different rat from that in Fig. 4.1). The TH-positive cells were located at the ventro-lateral edge of a graft, and the fibre shows multiple varicosities (arrowheads) along its length. Scale bar=20μm.
Figure 4.3. High magnification of a single TH-positive cell found in a graft from a 6-OHDA lesioned animal treated with L-DOPA and carbidopa treated. Several coarse branching processes, probably dendrites, can be seen.
4.3.3. $^3$H-Mazindol autoradiography

**Qualitative aspects**
In the intact striatum the density of $^3$H-mazindol binding varied between groups, and between animals of the same group. There was a lateral to ventro-medial gradient in density of binding. In the lesioned striatum, specific $^3$H-mazindol binding was greatly reduced as compared with the intact side in animals which had received unilateral 6-OHDA lesions followed by sham grafts, with or without L-DOPA and carbidopa treatment (groups A and B) (Figure 4.4A). A less marked reduction in binding was observed in the olfactory tubercle and nucleus accumbens. In animals which had received unilateral lesions followed by foetal DA cell grafts with or without treatment with L-DOPA and carbidopa (groups C and D), the most rostral sections (Level 1) showed marked reductions in specific $^3$H-mazindol binding on the lesioned/grafted side. At Level 2 (through graft placements) there were obvious areas of increased $^3$H-mazindol binding within the previously lesioned striatum (Figure 4.4B). Grafts were identified by $^3$H-mazindol binding in the dorsal, mid and ventral striatum and, occasionally, two grafts were identified in the same section. Generally, the central area of grafts was devoid of DA uptake sites (Figure 4.4B). Specific $^3$H-mazindol binding around grafts from animals treated with L-DOPA and carbidopa (group D) appeared greater than that in animals not receiving drug treatment (group C).

**Quantitative studies**

**Level 1: (Table 4.4)**
At Level 1, specific $^3$H-mazindol binding in the lesioned/grafted striatum of animals receiving sham grafts (groups A and B) was greatly reduced compared with the intact striatum. However, in animals receiving foetal grafts (groups C and D) the percentage reduction in binding on the lesioned side was less marked.
Figure 4.4. Colour photographs of autoradiograms of specific $^3$H-mazindol binding in striatal sections of animals with a unilateral 6-OHDA lesion followed by a sham graft (A) or a foetal DA cell graft (B).

A: Colour image of an autoradiograph showing the density of $^3$H-mazindol binding in a coronal section through Level 2 taken from an animal which had received a unilateral 6-OHDA lesion followed by a sham graft on the left and treatment with L-DOPA and carbidopa (group B). The colour scale shows the density of binding in nCi/mg tissue. The striatum, nucleus accumbens and olfactory tubercule can be seen clearly on the right. Note the lateral to ventro-medial decrease in density of specific binding on the intact side. On the left, the effects of the 6-OHDA lesion are obvious with virtual disappearance of $^3$H-mazindol binding in the striatum, nucleus accumbens and olfactory tubercule.

B: Colour image of an autoradiograph showing the density of $^3$H-mazindol binding in a coronal section through Level 2 taken from an animal receiving a unilateral 6-OHDA lesion followed by a foetal graft on the left and treated with L-DOPA and carbidopa (group D). The pattern of binding on the intact side (right side of image) is comparable to that in Fig. 4.4A. However, on the lesioned/grafted side, the graft tissue has resulted in a marked increase in the density of binding (compare lesioned side in Fig. 4.4A). Two grafts can be identified (dorsolateral and ventro-medial: *), and the density of binding is greatest between the 2 grafts, where it reaches similar levels to those found in the intact striatum. Note that the central areas of the grafts have low densities of ligand binding.
For the intact striatum at Level 1, neither L-DOPA and carbidopa treatment nor foetal DA cell grafts had any effect on the density of specific \(^{3}H\)-mazindol binding (using the raw data, 2-way ANOVA). Similarly, for the lesioned/grafted side, there was no effect of drug treatment or foetal graft on the density of binding. Re-analysis using a square-root transformation did not alter the findings for the intact side. However, the increase in \(^{3}H\)-mazindol binding resulting from the foetal graft on the lesioned side almost reached significance \((F(1,11)=4.38, p=0.06)\), even at this rostral level.

**Level 2: (Table 4.5)**

At Level 2, specific \(^{3}H\)-mazindol binding on the intact striatum at Level 2 ranged from 133-320 fmol/mg wet weight of tissue, and mean values were consistently higher in animals that had received treatment with L-DOPA and carbidopa (groups B and D), compared with untreated rats (groups A and C).

In the lesioned striatum of sham-grafted and foetal grafted animals, specific \(^{3}H\)-mazindol binding was reduced compared with the intact side \((p<0.001, \text{Student's } t\)-test). However, in animals receiving foetal grafts into the lesioned striatum (groups C and D), the density of \(^{3}H\)-mazindol binding was greater (means of 33 and 64.8 fmol/mg tissue respectively) than that in groups A or B which received sham grafts (means of 14.1 and 15.2 fmols/mg tissue). Expressed as a percentage of the intact side, the lesioned striatum of rats receiving foetal grafts contained up to 64% of the density of \(^{3}H\)-mazindol binding (Table 4.5), compared to a maximum of 11% in animals receiving sham grafts at this level. The density of \(^{3}H\)-mazindol binding in the grafted striata was higher in animals receiving foetal grafts plus L-DOPA (group D) than in those receiving foetal grafts alone (group C).
Table 4.4. The density of specific $^3$H-mazindol binding at Level 1 in the intact and 6-OHDA lesioned striatum of animals receiving sham grafts (groups A and B) or foetal grafts (groups C and D), either with (groups B and D) or without (groups A and C) treatment with L-DOPA and carbidopa.

<table>
<thead>
<tr>
<th>Group</th>
<th>Intact striatum (n=4)</th>
<th>Lesion/grafted striatum (n=3)</th>
<th>% Intact striatum (n=5)</th>
<th>Lesion/grafted striatum (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>204±14.2 (186-233)</td>
<td>8.9±0.7** (7.6-10)</td>
<td>4.3±0.3 (3.9-5.0)</td>
<td>4.3±0.3 (3.9-5.0)</td>
</tr>
<tr>
<td>B</td>
<td>215±42 (133-273)</td>
<td>17.1±4.4* (12-26)</td>
<td>8.1±1.4 (5.2-9.7)</td>
<td>8.1±1.4 (5.2-9.7)</td>
</tr>
<tr>
<td>C</td>
<td>249±31.1 (193-320)</td>
<td>23.4±6.5** (10-40)</td>
<td>10.6±3.6 (6-21)</td>
<td>10.6±3.6 (6-21)</td>
</tr>
<tr>
<td>D</td>
<td>259±12.5 (231-301)</td>
<td>49.7±18.5** (16-114)</td>
<td>18.0±6.4 (7-41)</td>
<td>18.0±6.4 (7-41)</td>
</tr>
</tbody>
</table>

* P<0.05 and ** P<0.01 lesioned/grafted side compared with intact side within each group, paired Student's t-test.

The values represent an average density for the whole cross-sectional area of striatum on the intact side and on the lesioned/grafted side in sections taken at Level 1, anterior to the sham or foetal graft placements. Values shown are mean ± 1 SEM, with the range in brackets. The last column shows the density of binding on the lesioned/grafted side expressed as a percentage of the intact side.
Table 4.5. The density of specific $^3$H-mazindol binding at Level 2 in the intact and 6-OHDA lesioned striatum of animals receiving a sham graft (groups A and B) or foetal graft (groups C and D), either with (groups B and D) or without (groups A and C) treatment with L-DOPA and carbidopa.

<table>
<thead>
<tr>
<th>Group</th>
<th>Intact striatum</th>
<th>Lesion/grafted striatum</th>
<th>% Intact striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>215±10.9</td>
<td>14.1±2.7*</td>
<td>6.1±1.0</td>
</tr>
<tr>
<td>(n=4)</td>
<td>(169-266)</td>
<td>(1.3-26)</td>
<td>(0.6-10.8)</td>
</tr>
<tr>
<td>B</td>
<td>247±9.1</td>
<td>15.2±2.5*</td>
<td>5.9±0.8</td>
</tr>
<tr>
<td>(n=3)</td>
<td>(220-300)</td>
<td>(3.3-29)</td>
<td>(1.4-10.4)</td>
</tr>
<tr>
<td>C</td>
<td>216±14.1</td>
<td>33.0±4.3*</td>
<td>16.0±2.7</td>
</tr>
<tr>
<td>(n=5)</td>
<td>(133-314)</td>
<td>(13-108)</td>
<td>(6.5-43)</td>
</tr>
<tr>
<td>D</td>
<td>244±12.1</td>
<td>64.8±10.5*</td>
<td>28.1±5.0</td>
</tr>
<tr>
<td>(n=6)</td>
<td>(146-320)</td>
<td>(14.0-131)</td>
<td>(2.7-64)</td>
</tr>
</tbody>
</table>

* $P<0.001$ compared with the intact side within each group, Student's paired t-test.

Values represent an average density over the whole cross-sectional area of striatum on the intact side and on the lesioned/grafted side in sections taken at Level 2, through the foetal or sham graft placements. Values shown are mean ± 1 SEM taken from 3 or 4 sections in each animal, with the range in brackets. The last column shows the density of binding on the lesion/grafted side expressed as a percentage of the intact side.
"Spot" readings taken directly over the region of the lesioned striatum containing the graft in animals from groups C and D revealed $^3$H-mazindol binding densities ranging from 72-300 fmols/mg wet weight tissue. The highest "spot" readings were found in foetal grafts in animals receiving L-DOPA and carbidopa treatment (group D). However, there was no significant difference between the mean values of the 2 groups (group C: mean 108.3±19.7 (SEM), range 72-140; group D: mean 163.5±23.8 (SEM), range 93-300; Student's t-test: t=1.7, df=7, p=0.1).

A 2-way ANOVA was performed on the raw data obtained at Level 2. For the intact striatum, treatment with L-DOPA and carbidopa produced a small (but not significant) increase in density of $^3$H-mazindol binding in groups B and D compared to groups A and C (F (1,11)=3.9, p=0.07). Using a square root transformation of the raw data, the increase became significant (F (1,11)=4.94, p<0.05). Implantation of foetal grafts into the lesioned striatum had no effect on binding on the intact side. For the lesioned striatum, a 2-way ANOVA performed on the raw data showed that a foetal DA cell graft (as in groups C and D) increased specific $^3$H-mazindol binding (F(1,11)= 8.62, p=0.014). In addition, treatment with L-DOPA and carbidopa was associated with an increase in the density of $^3$H-mazindol binding in the lesioned striatum of rats receiving foetal grafts, although this was not quite significant. Square root transformation of the data did not alter this finding.

4.4. DISCUSSION

These results indicate that treatment with L-DOPA (target dose 200 mg/kg/24hr) and carbidopa (target dose 25mg/kg /24hr), for 5 weeks in the drinking water does not impair the survival or fibre outgrowth of rat foetal VM grafts, as revealed by TH immunohistochemistry and specific binding of $^3$H-mazindol to brain slices.
TH Immunohistochemistry of host midbrain and foetal grafts

On the intact side of the mid-brain of rats with unilateral 6-OHDA lesions, the number and morphology of TH-immunoreactive cells in the SN and VTA were similar in both groups of animals receiving intra-striatal foetal grafts (groups C and D), indicating that treatment with L-DOPA and carbidopa did not have a cytotoxic effect on healthy DA cells in the intact host SN/VTA. Previous studies have shown no reduction in number or alteration in morphology of SN cells in normal animals treated chronically with high doses of L-DOPA (Sahakian et al 1980; Hefti et al 1981; Perry et al 1984). The cytoarchitecture of the intact SN and VTA in both groups of animals was comparable to that reported elsewhere in a study of normal rats (Hanaway et al 1970). However, there was a greater reduction of TH-positive cells in the VTA ipsilateral to the 6-OHDA lesion in animals treated with L-DOPA and carbidopa, although this was not quite significant. This observation suggests that chronic L-DOPA treatment may be harmful to cells previously exposed to this neurotoxin, and is discussed further in Chapter 6.

The effect of the unilateral 6-OHDA lesion was clearly demonstrated, with cell counts in the ipsilateral SN reduced by > 97% compared with the contralateral side in both groups of animals receiving foetal grafts. This was consistent with the rotational responses to apomorphine and (+)-amphetamine observed after the 6-OHDA lesion, and confirms that the subsequent behavioural recovery which followed implantation of foetal grafts (groups C and D) could not be attributed to incomplete denervation following the lesion, or to regeneration of host DA fibres. Cell counts in the ipsilateral VTA were also markedly reduced.

In animals receiving foetal VM grafts into the lesioned striatum (groups C and D), many TH-positive fibres and cells were present and the appearance of the grafts was
similar in the two groups, regardless of treatment with L-DOPA and carbidopa (group D). As noted by others (Jaeger 1985; Abrous et al 1988), the final form of the graft was influenced by its position within the host brain. Thus, grafts located close to the corpus callosum did not extend fibres into it, although extensive fibre outgrowth was observed into the surrounding denervated striatum (normal target tissue). TH-positive cells were often clustered at the dorsal or ventral pole of the graft, or around its periphery (Bjorklund et al 1983a, Jaeger 1985, Abrous et al 1988). The appearance of the TH-positive cells in the grafts was similar to that found in the host SN.

Importantly, there was no difference between the number of the TH-positive cells in grafts from animals treated with L-DOPA and carbidopa (group D) and those receiving foetal grafts alone (group C), indicating that the drug treatment had not impaired the survival of grafted tissue. There was, however, quite wide variation in cell number between animals even within the same group. Similarly, there was no evidence of stunting of fibre outgrowth from the grafts of L-DOPA and carbidopa treated animals, although this was not quantified using TH immunohistochemistry. TH-positive cell morphology within the grafts was not obviously affected by L-DOPA treatment.

Graft volumes varied even within the same rat where more than one graft was identified, as found previously (Bjorklund et al 1979, 1983a). However, when considered in the groups, graft volumes in rats receiving treatment with L-DOPA and carbidopa (group D) did not differ from those found in rats receiving foetal grafts alone (group C).

\textit{\textsuperscript{3}H-Mazindol autoradiography}

A quantitative estimate of the density of host nigrostriatal DA fibres and the fibre outgrowth from the grafts was made using \textit{\textsuperscript{3}H-mazindol autoradiography}. In the
intact striatum, the density of specific binding was homogeneous in most areas, apart from the lateral to ventro-medial gradient - which reflects the dopaminergic innervation from the SN (lateral striatum) and VTA (medial striatum) (Bjorklund and Lindvall 1986). In all animals receiving unilateral 6-OHDA lesions and sham-grafts (groups A and B), there was >90% depletion of specific ³H-mazindol binding in the striatum on the lesioned compared with the intact side. This is consistent with the rotational scores found in groups A and B during behavioural testing, which were indicative of a >98% reduction of striatal DA levels (Schmidt et al 1983), and the TH studies of SN cell counts presented here (>97% DA cell loss). Thus, in the sham-grafted animals, the density of ³H-mazindol binding appears to reflect the density of DA fibres remaining in the lesioned striatum.

The presence of a foetal DA graft in the lesioned striatum produced a marked increase in the density of specific ³H-mazindol binding in most cases, restoring levels to as high as 64% of the intact side when an average value over the whole striatum was considered. Using ³H-DA autoradiography in the mutant weaver mouse model, Doucet et al (1989b) found that foetal DA cell grafts produced a mean reinnervation amounting to 20-80% in the region immediately surrounding the graft. Similarly, in this study "spot" readings taken from the graft vicinity revealed densities of specific ³H-mazindol binding almost as high as those in the intact striatum.

Treatment with L-DOPA and carbidopa for 5 weeks following foetal grafts (group D) or sham grafts (group B), followed by a drug-free period of ~3 weeks before killing, was associated with an increase in the density of ³H-mazindol binding in the intact striatum compared with animals not receiving L-DOPA (groups A and C). It is possible that administration of L-DOPA and carbidopa and/or subsequent drug withdrawal resulted in an increase in uptake sites on
individual DA nerve terminals. Wiener et al (1989) found that chronic treatment with L-deprenyl resulted in an increase in the number of $^3$H-mazindol sites in the striatum. One consequence of both L-deprenyl-induced inhibition of monoamine oxidase activity (and DA uptake), and of chronic treatment with high doses of L-DOPA and carbidopa would be an increase in DA concentration in the synaptic space. This may result, by an unknown mechanism, in proliferation of DA uptake sites.

These results suggest that in vivo treatment of rats with this oral L-DOPA regime for 5 weeks does not reduce the survival of implanted foetal DA cell grafts. Fibre outgrowth from these grafts also appears to be unaffected. Steece-Collier et al (1990), using solid foetal VM tissue transplants, reported that chronic treatment with L-DOPA and carbidopa, using a twice daily ip injection regime, resulted in a reduction of DA cell size in the grafts and a patchy reduction of fibre outgrowth, although cell number was not affected. However, ip injection of L-DOPA and carbidopa has been shown previously to result in peak plasma levels of L-DOPA that are 5-6 times as high as those achieved when the same dose of L-DOPA is administered orally (Hare et al 1973).

A separate concern is that high levels of DA may, by acting on post-synaptic receptors, reduce the outgrowth of developing presynaptic fibres (Lankford et al 1988). In the present study, there was no evidence that fibre outgrowth, as quantified by $^3$H-mazindol autoradiography, was reduced by this L-DOPA treatment.
Chapter 5

EFFECTS OF A UNILATERAL 6-OHDA LESION, CHRONIC L-DOPA AND CARBIDOPA TREATMENT AND FOETAL VENTRAL MESENCEPHALIC GRAFTS ON THE DENSITIES OF D-1 AND D-2 DOPAMINE RECEPTORS IN STRIATAL SLICES AS REVEALED BY \(^{3}\text{H}-\text{SCH 23390}\) AND \(^{3}\text{H}-\text{SPIPERONE}\) AUTORADIOGRAPHY.
5.1. INTRODUCTION

In the experiment described in Chapter 3 it was found that treatment with L-DOPA and carbidopa did not interfere with the functional effects of foetal dopamine (DA) cell grafts, as revealed by reduction of drug-induced rotation. Another manifestation of foetal grafts which could be adversely affected by L-DOPA is their effect on DA receptor densities in the 6-OHDA lesioned striatum. In rats a unilateral 6-OHDA lesion of the nigrostriatal pathway produces an ipsilateral increase in striatal D-2 receptor density (Creese et al 1977, Guerin et al 1985, Savasta et al 1987a, Marshall et al 1989, Graham et al 1990). The changes in D-1 receptor density that follow a 6-OHDA lesion are more controversial (see section 5.4).

Theoretically, if DA delivery to the denervated striatal target neurones is restored, any changes in the density of D-1 and D-2 receptors consequent upon a lesion might be reversed. Foetal DA cell grafts have been shown to restore previously elevated D-2 receptor density resulting from 6-OHDA or MPP⁺ lesions of the nigrostriatal pathway in rats, to levels found in the intact striatum (Freed et al 1983; Sirinathsinghji et al 1990; Dawson et al 1991). Kaseda et al (1990), using the mutant weaver mouse model, found that foetal VM grafts prevented the increase in D-2 receptor density that normally develops in these animals by 6 months of age. Thus, foetal VM grafts restore denervation-related changes in D-2 receptors towards normal. However, the effect of L-DOPA treatment on this aspect of foetal graft function is unknown. In this study the effect of L-DOPA treatment on the ability of foetal VM grafts to restore lesion-induced changes in DA receptors has been investigated.

In addition, L-DOPA treatment itself may produce abnormalities in striatal DA receptors which might be responsible for L-DOPA-related dyskinesias in Parkinson's
disease (Mouradian et al 1988). Chronic L-DOPA treatment has been reported to reverse lesion-induced D-2 receptor supersensitivity (Reches et al 1984, Gropetti et al 1986, Parenti et al 1986), or further increase it (Rouillard et al 1987). The effect of L-DOPA treatment on D-1 receptors in lesioned rats is uncertain. In parkinsonian patients receiving a foetal graft, a situation exists of chronic striatal DA depletion, chronic L-DOPA treatment and the presence of foetal DA cells in the denervated striatum. It is important to know whether L-DOPA-related motor abnormalities are associated with changes in striatal DA receptors, and if so, whether those changes can be ameliorated by foetal DA cell grafts.

For these reasons, the effects of foetal VM grafts alone, L-DOPA treatment alone, and a combination of foetal grafts and L-DOPA treatment on the autoradiographic density of striatal D-1 and D-2 DA receptors was studied in 6-OHDA-lesioned rats. The animals used for this study were taken from the larger groups studied behaviourally in Chapter 3.

5.2. METHODS

5.2.1. Animals and drug treatment
The same rats, taken from the 4 large groups referred to in Chapter 3 (see section 3.2.4), were used for this experiment as were used for the \(^3\)H-mazindol autoradiography described in Chapter 4.

The groups of animals used were as follows:

Group A: 6-OHDA lesion + sham graft (n=4)
Group B: 6-OHDA lesion + sham graft + L-DOPA and carbidopa (n=4)
Group C: 6-OHDA lesion + foetal graft (n=5)
Group D: 6-OHDA lesion + foetal graft + L-DOPA and carbidopa (n=6)
Animals in the sham-grafted groups (A and B) were selected from the larger groups of rats studied in Chapter 3, on the basis of showing persistent high rates of ipsilateral rotation to (+)-amphetamine and contralateral rotation to apomorphine in the final test (test 3). All rats in the foetal-grafted groups (C and D) were selected on the basis of having shown a reduction of at least 80% of (+)-amphetamine-induced ipsilateral rotation in the final test (test 3, see section 3.3.3.2). All animals were killed approximately 8.5 weeks after foetal or sham graft surgery (i.e. within 2 days of the final apomorphine test (see Figure 2.20 and section 3.3.3), and approximately 3 weeks after L-DOPA treatment was stopped in groups B and D). Before killing, animals in groups B and D had received 5 weeks treatment with L-DOPA and carbidopa at target doses of 200mg/kg/24h and 25mg/kg/24h respectively, as described in section 2.5.

5.2.2. \textit{In vitro} binding of $^3$H-spiperone and $^3$H-SCH 23390 to striatal sections

\textit{Tissue preparation}

Rats were anaesthetised with pentobarbital (50mg/kg ip) and perfused transcardially with 300ml ice cold 5% dextrose saline. Brains were removed and 'snap' frozen in isopentane and mounted on cryostat chucks. Coronal sections (20μm) throughout the whole striatum were cut at -20°C using a Bright Instruments cryostat. Sections were thaw-mounted in duplicate onto subbed slides with alternate sections allocated to slides for total and non-specific binding, and then dried in a stream of cool air.

\textit{Binding studies}

Autoradiographic studies were supported by preliminary tissue swabbing studies to determine the kinetic and equilibrium characteristics of $^3$H-spiperone (for D-2 receptors) and $^3$H-SCH 23390 (for D-1 receptors) binding to striatal slices, according to the principles outlined by
Kuhar (1985). The results of these preliminary studies are shown in section 2.8.

For autoradiographic binding studies, adjacent sections (20μm thick) were incubated with $^3$H-spiperone or $^3$H-SCH 23390, as described in section 2.8.7. Autoradiograms were generated by apposing slide-mounted tissue sections to tritium sensitive film (tritium Hyperfilm, Amersham) at 4°C for 8 weeks for $^3$H-spiperone and 2 weeks for $^3$H-SCH 23390 together with Amersham tritium standards.

Computer assisted densitometry
Computerised densitometry (IBAS-2000; Kontron) was used to analyse the autoradiograms, as described in section 2.8.9. Quantitative measurements of optical density in the striatal slices were made from autoradiograms of serial coronal sections, taken at 3 levels along the rostro-caudal axis (Level 1: AP +0.7 from bregma, anterior to sham or foetal graft placements; Level 2: AP +0.2 - +0.3, through sites of sham or foetal graft placements; and Level 3: AP -1.0, posterior to sham or foetal graft placements) using the atlas of Paxinos and Watson (1982). For each section mean optical density readings were taken over the whole cross-sectional area of the right and left striata ('whole') and over the 4 quadrants of each striatum (dorsolateral (DL), ventrolateral (VL), dorsomedial (DM) and ventromedial (VM) on both right and left sides. Where a graft was visible in any section, the graft was carefully excluded from the area outlined by the cursor. This was to ensure that any changes in density of ligand binding reflected changes in the striatum surrounding the grafts, as ligand binding within the grafts (for both ligands) appeared to be consistently low. Readings for specific binding within the grafts themselves were obtained by outlining the graft with the cursor.

5.2.3. Statistics
A square root transformation of the raw data was used in
all the analyses, to reduce the effect of variation in level and spread. Comparisons of binding on the left and right striata within each group were made using a paired Student's t-test. Analysis of variance (ANOVA) was used for inter-group comparisons of binding in the intact striatum and lesioned/grafted striatum (with the intact side as covariate), followed by post hoc Dunnett's test.

5.3. RESULTS

5.3.1. Behavioural Assessment

Apomorphine-induced rotation (Table 5.1)
As described in Chapter 3, all 6-OHDA lesioned animals had shown contralateral rotation of >6/minute following administration of apomorphine (0.5mg/kg sc) and >8 ipsilateral rotations/minute in response to (+)-amphetamine (5mg/kg ip), when tested immediately before sham or foetal graft surgery (Tables 5.1 and 5.2, pre-graft score). When tested approximately 8 weeks after sham graft surgery (post-graft score in Table 5.1), contralateral rotation following administration of apomorphine (0.5mg/kg sc) in animals with a 6-OHDA lesion and sham grafts either with (group B) or without (group A) L-DOPA treatment was unchanged compared with the pre-graft score. In contrast, animals receiving foetal grafts with (group D) or without (group C) L-DOPA treatment displayed a 40-50% reduction in contralateral rotation. This reduction was significant in both groups of rats (t=4.06, df=4, p<0.05 for group C; and t=4.31, df=5, p=0.008 for group D). Whilst the foetal graft was associated with a significant reduction in apomorphine-induced contralateral rotation (ANOVA; F(1,16)=18.65, p=0.001), L-DOPA treatment was without effect, and there was no interaction of foetal graft and L-DOPA treatment on rotational rates.
Table 5.1. Rotational rates of 6-OHDA-lesioned rats following administration of apomorphine before and after a sham graft (groups A and B) or a foetal graft (groups C and D), either alone (groups A and C) or following treatment with L-DOPA and carbidopa (groups B and D)

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean no. rotations/min Pre-FG/SG</th>
<th>Mean no. rotations/min Post-FG/SG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (n=4)</td>
<td>10.3±0.4</td>
<td>10.2±0.7</td>
</tr>
<tr>
<td>Group B (n=4)</td>
<td>12.5±1.7</td>
<td>13.3±1.4</td>
</tr>
<tr>
<td>Group C (n=5)</td>
<td>12.1±1.8</td>
<td>*6.7±5.0</td>
</tr>
<tr>
<td>Group D (n=6)</td>
<td>13.8±3.4</td>
<td>**6.2±2.0</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01 compared to pre-graft score.

The net mean rotation rates (± SEM) per minute obtained over 30 minutes immediately following administration of apomorphine (0.5mg/kg sc) are shown. Positive values are rotations contralateral to the lesion. Tests were performed immediately prior to foetal graft (FG) or sham graft (SG) surgery and 8 weeks later (post-FG/SG). All animals had received a unilateral 6-OHDA lesion. Rats in groups A and B received a sham graft, whilst groups C and D received foetal VM grafts into the lesioned striatum. Groups B and D received 5 weeks treatment with L-DOPA and carbidopa immediately after FG/SG surgery.
(+)-Amphetamine-induced rotation (Table 5.2) Administration of (+)-amphetamine (5mg/kg ip) 6 weeks after sham graft surgery, to animals with (group B) or without (group A) L-DOPA treatment produced ipsilateral rotation rates similar to those observed prior to sham graft surgery. In contrast, animals receiving a foetal graft (group C) or a foetal graft followed by L-DOPA treatment (group D) showed a marked reduction in ipsilateral rotation to (+)-amphetamine, with most animals in both groups showing a net contralateral rotation. This reduction in rotation rates was significant (group C, t=6.97, df=4, p=0.001; group D, t=4.66, df=5, p<0.01; Student's t-test). A foetal graft was associated with a reduction in (+)-amphetamine induced ipsilateral rotation (ANOVA; F(1,16)=76.37, p<0.0001), whilst L-DOPA treatment was without effect, and there was no interaction of foetal graft and treatment with L-DOPA on rotation rates.

5.3.2. Autoradiographic studies of $^{3}$H-spiperone and $^{3}$H-SCH 23390 binding to striatal sections

5.3.2.1. Specific $^{3}$H-spiperone binding
General description
In the intact striatum in all groups, specific $^{3}$H-spiperone binding was high in the DL and VL quadrants at Levels 1 and 2, but specific $^{3}$H-spiperone binding was highest of all in the VL quadrant of posterior sections taken through Level 3. Neither L-DOPA treatment nor a foetal graft affected specific $^{3}$H-spiperone binding in the intact striatum. Readings over the whole cross-sectional area of the intact striatum at each level showed no obvious A-P gradient. In the lesioned striatum of animals with sham grafts, specific $^{3}$H-spiperone binding was markedly increased in the DL, VL and DM quadrants at Level 2 (Figure 5.1A). L-DOPA treatment did not affect this increase. In animals with a foetal graft in the lesioned striatum, specific $^{3}$H-spiperone binding was similar to that found in the intact side (Figure 5.1B).
Table 5.2. Rotational rates of 6-OHDA-lesioned rats following administration of (+)-amphetamine before and after a sham graft (groups A and B) or a foetal graft (groups C and D) either alone (groups A and C) or following treatment with L-DOPA and carbidopa (groups B and D)

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-FG/SG</th>
<th>Post-FG/SG</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n=4)</td>
<td>15.0±2.3</td>
<td>14.8±0.6</td>
</tr>
<tr>
<td>B (n=4)</td>
<td>15.7±6.0</td>
<td>17.6±4.4</td>
</tr>
<tr>
<td>C (n=5)</td>
<td>13.7±2.2</td>
<td>*-1.0±1.8</td>
</tr>
<tr>
<td>D (n=6)</td>
<td>18.1±5.1</td>
<td>*-2.3±0.5</td>
</tr>
</tbody>
</table>

*P<0.01 compared with pre-graft score.

The net mean rotational rate (± SEM) per minute obtained over 1 hour immediately following administration of (+)-amphetamine (5mg/kg ip) are shown. Positive values are ipsilateral to the lesion, and negative values are contralateral. Tests were performed immediately prior to foetal graft (FG) or sham graft (SG) surgery and 6 weeks later (post-FG/SG). All animals had received a unilateral 6-OHDA lesion. Rats in groups A and B received a sham graft, whilst groups C and D received a foetal ventral mesencephalic graft into the lesioned striatum. Groups B and D received 5 weeks treatment with L-DOPA and carbidopa immediately after FG/SG surgery.
Figure 5.1. Pseudocolour transformation of specific binding of 0.4nM $^3$H-spiperone to striatal sections in images derived from autoradiographs of coronal forebrain sections taken through Level 2.

The left side of each image is the 6-OHDA-lesioned and sham-grafted (5.1A) or foetal-grafted (5.1B) hemisphere. The colour scale shows binding in nCi/mg tissue.

5.1A. This section is taken from an animal in group B. In the lesioned and sham grafted striatum, binding density is increased compared with the intact side, and the increase in this particular example is most marked in the DL, VL and DM quadrants (arrowheads).

5.1B. This section is taken from an animal in group D. In this particular example, specific binding density in the lesioned and foetal-grafted hemisphere is restored to levels slightly below those found in the right (intact) striatum. An asterisk (*) marks the location of foetal grafts.
Quantitative studies

Level 1 (Table 5.3 and Figure 5.2A)

(a) Whole striatum
The density of specific $^3$H-spiperone binding sites in the intact striatum did not differ between groups (ANOVA). In the 6-OHDA-lesioned striatum a small increase in specific $^3$H-spiperone binding was observed in all 4 groups of animals compared with the intact side. However, the size of the increase varied markedly both within and between groups. The mean increase ranged from 8.9% in group D (lesion followed by foetal graft and chronic L-DOPA treatment) to 25.1% in group A (lesion followed by sham graft). The increase compared to the intact side reached significance only in the latter group (group A, $t=6.29$, df=3, $p<0.05$; Student's t-test). There was no effect of the foetal graft or L-DOPA and carbidopa treatment on density of $^3$H-spiperone binding sites in the whole lesioned striatum at this level (ANOVA).

(b) Regional density
Specific $^3$H-spiperone binding in the 4 quadrants of the intact striatum did not differ between groups. In the lesioned striatum, animals receiving a 6-OHDA lesion followed by a sham graft (group A) showed a significant increase in specific $^3$H-spiperone binding compared to the intact side in the DL and DM quadrants. In animals with a 6-OHDA lesion followed by a sham graft and L-DOPA treatment (group B), small, non-significant increases were observed in all quadrants. In animals receiving a 6-OHDA lesion followed by a foetal graft (group C), small, non-significant increases in specific binding were observed in all quadrants (except DM for group C, where a small decrease was found). In animals receiving a 6-OHDA lesion followed by a foetal graft and L-DOPA treatment (group D), small, non-significant increases in specific $^3$H-spiperone binding were found in all quadrants, but the increase in the DM quadrant was substantially lower than that found in animals receiving sham grafts.
Table 5.3. The autoradiographic density of specific \(^3\)H-spiperone binding to striatal slices taken through Level 1 in the intact (Int.) and 6-OHDA-lesioned (Les.) striatum of animals receiving a sham graft (groups A and B) or a foetal graft (groups C and D), either with (groups B and D) or without (groups A and C) treatment with L-DOPA and carbidopa.

<table>
<thead>
<tr>
<th></th>
<th>Whole</th>
<th>DL</th>
<th>VL</th>
<th>DM</th>
<th>VM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=4)</td>
<td>52.9</td>
<td>65.3</td>
<td>59.0</td>
<td>75.7</td>
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</tr>
<tr>
<td>Group B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=4)</td>
<td>61.9</td>
<td>77.0</td>
<td>73.5</td>
<td>81.2</td>
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</tr>
<tr>
<td>Group C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=5)</td>
<td>56.1</td>
<td>64.2</td>
<td>62.5</td>
<td>67.1</td>
<td>57.2</td>
</tr>
<tr>
<td>Group D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=6)</td>
<td>57.3</td>
<td>60.4</td>
<td>58.4</td>
<td>62.2</td>
<td>62.4</td>
</tr>
</tbody>
</table>

*P<0.05 compared with intact side, Student’s t test.

Values shown are the mean +SEM for the whole cross-sectional area of the striatum (whole) and for each quadrant (DL=dorsolateral; VL=ventrolateral; DM=dorsomedial; VM=ventromedial) in sections taken through Level 1, anterior to the sham or foetal graft placements. All animals had received a unilateral 6-OHDA lesion. Rats in groups A and B received a sham graft, whilst groups C and D received a foetal VM graft into the lesioned striatum. Sham- or foetal-graft surgery was followed in groups B and D by 5 weeks treatment with L-DOPA and carbidopa.
Table 5.4. The autoradiographic density of specific $^3$H-spiperone binding to striatal slices taken through Level 2 in the intact (Int.) and 6-OHDA-lesioned (Les.) striatum of animals receiving a sham graft (groups A and B) or a foetal graft (groups C and D), either with (groups B and D) or without (groups A and C) treatment with L-DOPA and carbidopa.

<table>
<thead>
<tr>
<th></th>
<th>Whole</th>
<th>DL</th>
<th></th>
<th>VL</th>
<th></th>
<th>DM</th>
<th></th>
<th>VM</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>46.3</td>
<td>*65.5</td>
<td>66.1</td>
<td>75.2</td>
<td>64.8</td>
<td>*79.3</td>
<td>51.4</td>
<td>*65.2</td>
<td>46.2</td>
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<tr>
<td>(n=4)</td>
<td></td>
<td></td>
<td>+17.4</td>
<td>+12.8</td>
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</tr>
<tr>
<td>Group B</td>
<td>56.3</td>
<td>*67.7</td>
<td>66.2</td>
<td>78.2</td>
<td>67.8</td>
<td>*90.7</td>
<td>49.5</td>
<td>*66.5</td>
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<td>+6.5</td>
<td>+10.7</td>
<td>+9.7</td>
<td>+10.5</td>
<td>+16.5</td>
<td>+4.1</td>
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<td>Group C</td>
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<td>75.2</td>
<td>72.5</td>
<td>49.0</td>
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<td>+2.7</td>
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<td>+3.5</td>
<td>+2.9</td>
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<tr>
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<td>66.2</td>
<td>58.3</td>
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<td>+3.7</td>
<td>+2.9</td>
<td>+3.5</td>
<td>+3.2</td>
<td>+4.4</td>
</tr>
</tbody>
</table>

*P<0.05 compared with intact side, Student's t-test.

Values shown are the mean $\pm$SEM for the whole cross-sectional area of the striatum (whole) and for each quadrant (DL=dorsolateral; VL=ventrolateral; DM=dorsomedial; VM=ventromedial) in sections taken through Level 2, through sham or foetal graft placements. All animals had received a unilateral 6-OHDA lesion. Rats in groups A and B received a sham graft, whilst groups C and D received a foetal VM graft. Sham- or foetal-graft surgery was followed in groups B and D by 5 weeks treatment with L-DOPA and carbidopa.
Table 5.5. The autoradiographic density of specific $^3$H-spiperone binding to striatal slices taken through Level 3 in the intact (Int.) and 6-OHDA-lesioned (Les.) striatum of animals receiving a sham graft (groups A and B) or a foetal graft (groups C and D), either with (groups B and D) or without (groups A and C) treatment with L-DOPA and carbidopa.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>51.6 52.4</td>
<td>52.5 58.5</td>
<td>72.8 74.7</td>
<td>46.7 50.4</td>
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<tr>
<td>(n=4)</td>
<td>+7.9 +4.9</td>
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<td>+10.2 +7.3</td>
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<td>Group B</td>
<td>47.4 57.3</td>
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<td>72.5 89.8</td>
<td>40.5 50.0</td>
</tr>
<tr>
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<td>+11.9 +7.5</td>
<td>+13.6 +18.7</td>
<td>+7.9 +14.0</td>
</tr>
<tr>
<td>Group C</td>
<td>51.2 53.3</td>
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<td>82.2 86.2</td>
<td>44.7 46.1</td>
</tr>
<tr>
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<td>+4.9 +6.5</td>
<td>+2.6 +4.4</td>
<td>+9.6 +11.9</td>
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</tr>
<tr>
<td>Group D</td>
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<td>87.2 78.0</td>
<td>47.2 46.1</td>
</tr>
<tr>
<td>(n=6)</td>
<td>+6.0 +3.3</td>
<td>+9.0 +9.1</td>
<td>+11.8 +3.2</td>
<td>+6.2 +3.7</td>
</tr>
</tbody>
</table>

*P<0.05 compared with intact side, Student's t-test.

Values shown are the mean ±SEM for the whole cross-sectional area of the striatum (whole) and for each quadrant (DL=dorsolateral; VL=ventrolateral; DM=dorsomedial; VM=ventromedial) in sections taken through Level 3, posterior to the sham or foetal graft placements. All animals had received a unilateral 6-OHDA lesion. Rats in groups A and B received sham grafts, whilst groups C and D received foetal VM grafts into the lesioned striatum. Sham- or foetal-graft surgery was followed in groups B and D by 5 weeks treatment with L-DOPA and carbidopa.
Figure 5.2

Per cent binding difference

Striatal region

(a)

(b)

(c)

for 3H-spiroperone at level 1

for 3H-spiroperone at level 2

for 3H-spiroperone at level 3
Figure 5.2. The percentage difference in specific $^3$H-spiperone binding density between the left (6-OHDA-lesioned and sham- or foetal-grafted) and right (intact) striatum is shown for each of the 3 Levels analysed, for all groups of animals.

Striatal regions at the 3 rostro-caudal Levels are depicted in schematic diagrams. Increase in specific D-2 binding in the lesioned striatum compared with the intact striatum is depicted by bars extending above the zero line, and reduction by bars below the zero line. An asterisk (*) indicates a significant difference (p<0.05, Student's t-test) between the intact and lesioned/grafted sides of the brain, based on the square root of the mean absolute values (Student's t-test).

Key:
Group A (6-OHDA lesion + sham graft): [image]
Group B (6-OHDA lesion + sham graft + L-DOPA treatment): [image]
Group C (6-OHDA lesion + foetal graft): [image]
Group D (6-OHDA lesion + foetal graft + L-DOPA treatment): [image]
A 2-way ANOVA was performed separately on the data for each quadrant in the lesioned/grafted striatum, using the intact side as covariate. A reduction of D-2 binding was observed in the DM quadrant of the lesioned striatum due to the foetal graft (F(1,15)=6.65, p=0.026; post hoc Dunnett's test, t=2.8, df=14, p=0.015), but not in the DL, VM or VL quadrants. L-DOPA treatment was without effect on binding density in any quadrant.

Level 2 (Table 5.4 and Figure 5.2B)
(a) Whole striatum
As at Level 1, in the intact striatum there was no difference in the density of specific \(^3\)H-spiperone binding between the groups at Level 2 (ANOVA). In the lesioned striatum, animals receiving a sham graft alone (group A) or followed by L-DOPA treatment (group B) showed an increase in specific \(^3\)H-spiperone binding over the striatum as a whole (compared with the intact side). By contrast, in animals receiving a 6-OHDA lesion followed by a foetal graft alone (group C) or with L-DOPA treatment (group D), the density of specific \(^3\)H-spiperone binding in the lesioned striatum was slightly lower than that found in the intact side (p>0.05). Comparison of the binding densities in the lesioned/grafted side in the different groups (2-way ANOVA) showed that the foetal graft produced a decrease in binding on the lesioned side (F(1,14)=84.07, p<0.0001; post hoc Dunnett's test, t=9.72, df=15, p<0.0001). L-DOPA treatment was without effect.

Specific \(^3\)H-spiperone binding within the grafts of animals in groups C and D was consistently lower than that in the surrounding striatum. Specific \(^3\)H-spiperone binding within the grafts were similar in the two groups (group C = 22.8±2.6 fmol/mg; group D = 25.5±2.8 fmol/mg).

(b) Regional density
Specific \(^3\)H-spiperone binding in the 4 quadrants of the intact striatum did not differ between the groups. In the
lesioned striatum, animals receiving a unilateral lesion followed by a sham graft alone (group A) or with L-DOPA treatment (group B) showed an increase in specific $^3$H-spiperone binding in the VL and DM quadrants; group A also showed an increase in the DL quadrant. By contrast, in animals receiving foetal grafts alone (group C) or with L-DOPA treatment (group D), specific binding in the lesioned striatum was reduced ($p>0.05$) below that found in the contralateral intact striatum. A 2-way ANOVA revealed a reduction of specific $^3$H-spiperone binding sites in the lesioned striatum due to the foetal graft in all quadrants except VM (DL: $F(1,14)=17.35\text{ p}=0.002$; post hoc Dunnett's test, $t=4.31$, df=15, $p<0.0001$; VL: $F(1,14)=62.8\text{ p}<0.0001$; post hoc Dunnett's test, $t=7.28$, df=15, $p<0.0001$; DM: $F(1,15)=12.73\text{ p}=0.004$; post hoc Dunnett's test, $t=4.51$, df=15, $p<0.0001$). L-DOPA treatment was without effect.

**Level 3 (Table 5.5 and Figure 5.2C)**

(a) **Whole striatum**
The density of specific $^3$H-spiperone binding on the intact side again did not differ between groups (ANOVA). In addition, there was no difference in specific $^3$H-spiperone binding between the intact and lesioned sides for any group (paired t-tests). Neither foetal graft nor treatment with L-DOPA and carbidopa affected the density of specific binding found in the intact or lesioned/grafted striatum (ANOVA).

(b) **Regional density**
For all groups except group B (where an increase was found in the VL quadrant of the lesioned striatum) there was no difference in specific $^3$H-spiperone binding between the right and left striata for any quadrant.

**Direction of change**
The direction of change in $^3$H-spiperone binding in the lesioned striatum compared with the intact side at each level is summarised in Table 5.6.
Table 5.6. Summary of the direction of change in specific $^3$H-spiperone binding in the lesioned striatum compared with the intact side.

<table>
<thead>
<tr>
<th>Level</th>
<th>Whole</th>
<th>DL 1</th>
<th>DL 2</th>
<th>DL 3</th>
<th>VL 1</th>
<th>VL 2</th>
<th>VL 3</th>
<th>DM 1</th>
<th>DM 2</th>
<th>DM 3</th>
<th>VM 1</th>
<th>VM 2</th>
<th>VM 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
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<td>-</td>
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<td>-</td>
<td>↑↑</td>
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<tr>
<td>Group B</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Group C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group D</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The direction of change of specific $^3$H-spiperone binding in the lesioned/grafted striatum compared with the corresponding region of the intact side is shown. The changes found at each AP Level (Levels 1-3) are shown for the striatum as a whole and for each of the 4 quadrants. All animals had received a unilateral 6-OHDA lesion. Rats in groups A and B received sham grafts, whilst groups C and D received foetal VM grafts into the lesioned striatum. Sham- or foetal-graft surgery was followed in groups B and D by 5 weeks treatment with L-DOPA and carbidopa.
5.3.2.2. Specific $^3$H-SCH 23390 binding

General description
In the intact striatum in all groups specific $^3$H-SCH 23390 binding was greatest in the DL and VL quadrants at Level 1, and in the VL quadrant at Levels 2 and 3. In all groups, specific $^3$H-SCH 23390 binding density over the whole cross-sectional area of the intact striatum showed a rostro-caudal gradient (greatest in the anterior striatum [Level 1]).

A 6-OHDA lesion produced inconsistent changes in specific binding in the most posterior sections and also, though to a lesser extent, in the anterior sections. The only consistent changes occurred in the mid-body of the striatum (Level 2), where the lesion caused a small reduction in specific binding in the lateral quadrants (Figure 5.3A). The pattern and density of specific $^3$H-SCH 23390 binding were not affected by either the foetal grafts (Figure 5.3B) or by treatment with L-DOPA and carbidopa.

Quantitative description
Level 1 (Table 5.7 and Figure 5.4A)
(a) Whole striatum
The density of specific $^3$H-SCH 23390 binding in the intact striatum did not differ significantly between the groups, although it tended to be lower in animals which had received a foetal graft into the denervated striatum. In the lesioned striatum, specific binding was lower than in the intact striatum in all groups, but the difference was not significant in any. A foetal graft and/or treatment with L-DOPA and carbidopa did not affect specific $^3$H-SCH 23390 binding in the lesioned striatum (ANOVA).

(b) Regional density at Level 1
Specific binding in the lesioned striatum tended to be lower than in the intact striatum in all quadrants except VM in all groups. However, the difference was significant
Figure 5.3. Pseudocolour transformation of specific binding of 0.2nM $^3$H-SCH 23390 to striatal sections in images derived from autoradiographs of coronal forebrain sections taken through Level 2.

The left side of each image is the 6-OHDA-lesioned and sham-grafted (5.3A) or foetal-grafted (5.3B) hemisphere. The colour scale shows binding in nCi/mg tissue.

5.3A. This section is taken from an animal in group B. In this particular example, specific binding density is decreased in the DL and, to a lesser extent, in the VL quadrants of the lesioned and sham-grafted striatum (arrowheads) compared with the intact side. However, when all rats in each group were considered, the decrease in the lesioned striatum was most marked in the VL quadrant. Changes in other quadrants of the lesioned striatum were inconsistent. Thus, this section reveals an increase in the DM quadrant of the lesioned striatum, but this was not a consistent or significant finding.

5.3B. This section is taken from an animal in group D. Specific binding in the 6-OHDA-lesioned striatum containing foetal grafts (asterisks) is seen to be lower than that in the intact striatum, most obviously so in the DL and VL quadrants. In this particular example the reduction in the VL quadrant appears to be even lower than that found in the VL quadrant of the sham-grafted striatum (5.3A), but this was not a consistent or significant difference when all animals in the groups were considered.
Table 5.7. The autoradiographic density of specific $^3$H-SCH 23390 binding to striatal slices taken through Level 1 in the intact (Int.) and 6-OHDA-lesioned (Les.) striatum of animals receiving a sham graft (groups A and B) or a foetal graft (groups C and D), either with (groups B and D) or without (groups A and C) treatment with L-DOPA and carbidopa.

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
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<tbody>
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<td>144.0</td>
<td>122.0</td>
<td>140.0</td>
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</tr>
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<tr>
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</tr>
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<td>+9.5</td>
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</tr>
</tbody>
</table>

*P<0.05 compared with intact side, Student's t-test.

Values shown are the mean ±SEM for the whole cross-sectional area of the striatum (whole) and for each quadrant (DL=dorsolateral; VL=ventrolateral; DM=dorsomedial; VM=ventromedial) in sections taken through Level 1, anterior to the sham or foetal graft placements. All animals had received a unilateral 6-OHDA lesion. Rats in groups A and B received sham grafts, whilst groups C and D received foetal VM grafts into the lesioned striatum. Sham- or foetal-graft surgery was followed in groups B and D by 5 weeks treatment with L-DOPA and carbidopa.
Table 5.8. The autoradiographic density of specific $^3$H-SCH 23390 binding to striatal slices taken through Level 2 in the intact (Int.) and 6-OHDA-lesioned (Les.) striatum of animals receiving a sham graft (groups A and B) or a foetal graft (groups C and D), either with (groups B and D) or without (groups A and C) treatment with L-DOPA and carbidopa.

<table>
<thead>
<tr>
<th>Group</th>
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<th>VL</th>
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</tr>
<tr>
<td>D</td>
<td>106.2 **93.2</td>
<td>99.2 **93.3</td>
<td>110.5 **98.9</td>
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<td>89.5</td>
</tr>
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*P=0.06 and **P<0.05 compared with intact side, Student's t-test.

Values shown are the mean +SEM for the whole cross-sectional area of the striatum (whole) and for each quadrant (DL=dorsolateral; VL=ventrolateral; DM=dorsomedial; VM=ventromedial) in sections taken through Level 2, anterior to the sham or foetal graft placements. All animals had received a unilateral 6-OHDA lesion. Rats in groups A and B received sham grafts, whilst groups C and D received a foetal VM grafts into the lesioned striatum. Sham- or foetal-graft surgery was followed in groups B and D by 5 weeks treatment with L-DOPA and carbidopa.
Table 5.9. The autoradiographic density of specific $^3$H-SCH 23390 binding to striatal slices taken through Level 3 in the intact (Int.) and 6-OHDA-lesioned (Les.) striatum of animals receiving a sham graft (groups A and B) or a foetal graft (groups C and D), either with (groups B and D) or without (groups A and C) treatment with L-DOPA and carbidopa.

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<th></th>
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<th>Int. L</th>
<th>Int. V</th>
<th>Int. M</th>
<th>Int. D</th>
<th>Int. L</th>
<th>Int. V</th>
<th>Int. M</th>
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<td>93.2</td>
<td>86.7</td>
<td>94.6</td>
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<td>(n=4)</td>
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<td>+9.5</td>
<td>+6.8</td>
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<tr>
<td><strong>Group B</strong></td>
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<td>93.7</td>
<td>96.4</td>
<td>88.2</td>
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<td>109.4</td>
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<td>+5.6</td>
<td>+19.7</td>
<td>+5.5</td>
<td>+9.5</td>
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<td>+11.6</td>
<td>+9.8</td>
<td>+10.9</td>
<td>+4.8</td>
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</table>

Values shown are the mean $+SEM$ for the whole cross-sectional area of the striatum (whole) and for each quadrant (DL=dorsolateral; VL=ventrolateral; DM=dorsomedial; VM=ventromedial) in sections taken through Level 3, anterior to the sham or foetal graft placements. All animals had received a unilateral 6-OHDA lesion. Rats in groups A and B received sham grafts, whilst groups C and D received a foetal VM grafts into the lesioned striatum. Sham- or foetal-graft surgery was followed in groups B and D by 5 weeks treatment with L-DOPA and carbidopa.
Figure 5.4. The percentage difference in specific $^3$H-SCH 23390 binding density between the left (6-OHDA-lesioned and sham or foetal grafted) and right (intact) striatum is shown for each of the 3 Levels analysed, for all groups of animals.

Striatal regions at the 3 rostro-caudal Levels are depicted in schematic diagrams. Reduction of D-1 binding in the lesioned compared with the intact striatum is depicted by bars extending below the zero line, and increase by bars above the zero line. An asterisk (*) indicates a significant difference (p<0.05, Student's t-test) between the intact and lesioned/grafted sides of the brain based on the square root of the mean absolute values.

Key:
Group A (6-OHDA lesion + sham graft): 
Group B (6-OHDA lesion + sham graft + L-DOPA treatment): 
Group C (6-OHDA lesion + foetal graft): 
Group D (6-OHDA lesion + foetal graft + L-DOPA treatment):
only for animals receiving a 6-OHDA lesion followed by a foetal graft (group C), and then only in the lateral quadrants. A foetal graft and L-DOPA treatment were without effect on the levels of binding found in the lesioned striatum (ANOVA).

Level 2 (Table 5.8 and Figure 5.4B)
(a) Whole striatum
As at Level 1, the density of specific $^3$H-SCH 23390 binding over the whole cross-sectional area of the intact striatum at Level 2 appeared to be lower in animals receiving foetal grafts into the lesioned striatum (groups C and D) than in those receiving sham grafts (groups A and B), but this was not significant (ANOVA). In the lesioned striatum, all groups of animals demonstrated a decrease in specific $^3$H-SCH 23390 binding in the whole cross-sectional area of the lesioned/grafted striatum compared with the intact side. The difference was significant, however, only in animals receiving a 6-OHDA lesion followed by a foetal graft and chronic L-DOPA treatment (group D, \(t=2.79, \ df=5, \ p=0.039\)), and almost reached significance in animals receiving a lesion followed by a sham graft (group A, \(t=4.0, \ df=2, \ p=0.057\)). However, specific binding on the lesioned side was not affected by the presence of a foetal graft and/or L-DOPA treatment (ANOVA).

Specific $^3$H-SCH 23390 binding within the foetal grafts was much lower than that in the surrounding striatum (of the order of 10%), and did not differ between the 2 groups receiving foetal grafts (group C, mean = 10.9$\pm$2.6 fmol/mg; group D mean = 9.8$\pm$2.5 fmol/mg).

(b) Regional density
All groups of animals showed a small reduction in specific $^3$H-SCH 23390 binding in all quadrants of the lesioned striatum compared with the intact side. These differences were significant (\(p<0.05\)) in relation to the DL and VL quadrants in group A (lesion followed by sham graft),
Table 5.10. Summary of the direction of changes in $^3$H-SCH 23390 binding in the lesioned striatum compared with the intact side.

<table>
<thead>
<tr>
<th>Level</th>
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<th>VL</th>
<th>DM</th>
<th>VM</th>
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<td>1 2 3</td>
<td>1 2 3</td>
<td>1 2 3</td>
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<td>- ↓ -</td>
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<td>Group D</td>
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</tbody>
</table>

The direction of change of specific $^3$H-SCH 23390 binding in the lesioned/grafted striatum compared with the corresponding region of the intact side is shown. The changes found at each AP level (Levels 1-3) are shown for the striatum as a whole and for each of the 4 quadrants. All animals had received a unilateral 6-OHDA lesion. Rats in groups A and B received sham grafts, whilst groups C and D received foetal VM grafts into the lesioned striatum. Sham- or foetal-graft surgery was followed in groups B and D by 5 weeks treatment with L-DOPA and carbidopa.
group C (lesion followed by foetal graft), and group D (lesion followed by foetal graft and L-DOPA treatment), and nearly significant in relation to the DL and VL quadrants in group B (lesion followed by sham graft and L-DOPA treatment). The reductions found in the DM and VM quadrants were not significant in any of the groups. A 2-way ANOVA showed no effect of foetal graft and/or L-DOPA treatment on the binding in any quadrant.

**Level 3 (Table 5.9 and Figure 5.4C)**
In all groups the density of specific $^{3}$H-SCH 23390 binding did not differ between the right and left striata either over the striatum as a whole or in any quadrant.

**Direction of Change**
The direction of change in $^{3}$H-SCH 23390 binding in the lesioned striatum compared with the intact side at each level is summarised in Table 5.10.

5.4. DISCUSSION

5.4.1. BEHAVIOURAL RESPONSES
High rates of rotation in response to apomorphine and (+)-amphetamine were observed in all tests in animals with 6-OHDA lesions and sham grafts regardless of treatment with L-DOPA. However, as described in Chapter 3, and in confirmation of earlier studies (Bjorklund et al 1980a,b; Dunnett et al 1983), foetal VM grafts reduced apomorphine-induced contralateral rotation, and abolished (+)-amphetamine-induced ipsilateral rotation. Concurrent administration of L-DOPA to grafted animals did not interfere with this behavioural recovery.

5.4.2. AUTORADIOGRAPHIC STUDIES
The present autoradiographic studies were undertaken using only a single concentration of each of the ligands, $^{3}$H-spiiperone and $^{3}$H-SCH 23390, being equivalent to the Kd for each ligand as obtained from preliminary biochemical
studies (see section 2.8). The changes in specific binding that were observed in the autoradiographs cannot categorically be interpreted as changes in density of the respective binding sites, but might have resulted from changes in the affinity of ligand binding to the binding sites. However, previous studies assessing the effects of a 6-OHDA lesion on striatal DA receptors have shown the changes to be due to changes in $B_{\text{max}}$ and not $K_d$ (Neve et al 1984, Hyttel and Arnt 1987, Marshall et al 1989). Joyce (1991a,b), who found similar lesion-induced changes in D-1 and D-2 receptor binding to the present results, also demonstrated that these reflected changes in $B_{\text{max}}$ and not in $K_d$ (Joyce 1991a,b). For the purposes of this study, therefore, the autoradiographic density will be assumed to reflect binding density of the receptor sites. On this basis, the main findings of this study were:

1. The density of D-2 receptors, as revealed by specific $^3\text{H}$-spiperone binding in striatal slices, was increased in the 6-OHDA lesioned striatum, most markedly in the VL and DM quadrants at Level 2.

2. Foetal VM grafts reversed the increase in $^3\text{H}$-spiperone binding in the lesioned striatum, but chronic L-DOPA treatment alone did not. Chronic L-DOPA treatment did not interfere with the ability of the grafts to restore D-2 binding density to levels found in the intact striatum.

3. The density of D-1 receptors, as revealed by specific $^3\text{H}$-SCH 23390 binding in striatal slices, was reduced in the VL quadrant of the lesioned striatum at Level 2. However, inconsistent, non-significant, changes were found in other areas of the lesioned striatum. Neither a foetal graft nor chronic L-DOPA treatment, nor a combination of the two altered the lesion-induced changes.

(i) $^3\text{H}$-spiperone binding
The density of D-2 binding sites in the intact striatum
was greatest in the lateral quadrants at all levels. A similar pattern has been observed by others in autoradiographic studies (Savasta et al 1987a, Filloux et al 1988, Joyce 1991a,b).

**Effect of the lesion**
A chronic unilateral 6-OHDA lesion caused a consistent increase in the density of D-2 sites in the lesioned striatum, most marked in the VL and DM quadrants at Level 2. This is in broad agreement with previous autoradiographic studies (Savasta et al 1987b, Beresford et al 1988, Marshall et al 1989, Graham et al 1990, Joyce 1991a,b), although the magnitude of the reported increases has varied. Graham et al (1990) found increases of as much as 67% in the lateral quadrants of the body of the striatum. Those authors suggested that the discrepancies between different studies in the size of the observed changes might be due to differences in completeness of the lesion. However, the animals used in the present study showed rotational rates in behavioural tests indicative of >99% loss of striatal DA (Schmidt et al 1982), yet the increase in D-2 density never exceeded 33% in any quadrant. Other factors which could explain the differences in observed density of D-2 binding sites are the use of different ligands, variation in the position of the 6-OHDA lesion, differences in the time elapsed since lesioning, and differences in ages of the animals.

**Effect of L-DOPA treatment**
Administration of L-DOPA and carbidopa to rats with a unilateral 6-OHDA lesion did not alter specific $^3$H-spiperone binding in the intact striatum, and was without effect on the lesion-induced increase in D-2 receptor binding in the lesioned striatum. Previous studies of the effect of L-DOPA on D-2 receptors in the intact rat striatum using in vitro homogenates have revealed no change in density (Jackson et al 1983), an increase (Rouillard et al 1987), or a small decrease (Rouillard et
al 1987). These studies are made difficult to compare not only by differences in protocol, but also by differences in L-DOPA treatment regime (including dosage, route of administration and duration of treatment). Differences in L-DOPA regime can profoundly affect the subsequent behavioural response to apomorphine (Juncos et al 1989) and D-1 agonists (Weick et al 1990), and conceivably could also be responsible for observed differences in D-2 receptor density.

**Effect of foetal VM grafts**

Foetal VM grafts implanted into the 6-OHDA-lesioned striatum restored D-2 receptor density to levels found in the intact striatum. This effect of the grafts was not altered by chronic L-DOPA treatment. Reduction of D-2 receptor supersensitivity by foetal grafts may reflect chronic DA release by the implanted cells. If this were so, the observation that L-DOPA treatment did not interfere with the ability of grafts to reduce D-2 receptor density increased by a 6-OHDA lesion may therefore suggest that chronic DA release by the grafts was not adversely affected by this drug treatment.

The foetal grafts themselves, as identified on autoradiograms, appeared to have low densities of specific $^3$H-spiperone binding. Dawson et al (1991) reported similar findings. DA cell dendrites and cell bodies may be the main sites of DA receptors in the adult SN (Bouthenet et al 1987), so it is reasonable to suppose that these receptors are located similarly in the grafted foetal DA cells. If this is so, the present findings may not be surprising, particularly bearing in mind the observation that TH-positive cells in the grafts appeared to be located mainly at the graft periphery (Chapters 4 and 6). Also, the autoradiograms were not accompanied by parallel studies for TH-immunohistochemistry, so the precise host-graft border was uncertain. It is possible that areas in the immediate vicinity of the graft which
were designated as striatum by the cursor outlining technique may have contained some grafted material. A similar explanation may also apply to the low specific $^3$H-SCH 23390 binding observed within the grafts (similar finding reported by Dawson et al, 1991).

(ii) $^3$H-SCH 23390 binding

Effect of the lesion, L-DOPA treatment and foetal graft

A 6-OHDA lesion produced a small ipsilateral reduction in D-1 binding density in the VL quadrant at Level 2. This result is consistent with some other autoradiographic studies of the effects of a 6-OHDA lesion on D-1 density (Marshall et al 1989; Joyce 1991a,b). However, some reports of the effect of a 6-OHDA lesion on the density of D-1 receptors have revealed persistent (Buonamici et al 1986, Porceddu et al 1987, Dawson et al 1991), or transient (Caccia et al 1988; Herve et al 1989) increases in D-1 receptor density, and others have found no or inconsistent changes in D-1 receptor density (Altar et al 1987; Savasta et al 1987a, 1988; Graham et al 1990). Some of the reported differences in D-1 binding following a lesion could be explained by differences in the age of the lesion at the time of study or its exact location (Herve et al 1989), or even by the units in which the results are expressed (Ohta et al 1988).

Neither the L-DOPA treatment nor foetal grafts (nor a combination of the two) produced any alteration in the lesion-induced reduction of D-1 binding at Level 2, nor did they affect the binding density at the other levels. Dawson et al (1991) reported that a foetal nigral graft restored a 6-OHDA lesion-induced increase in D-1 (and D-2) receptor density in the striatum to intact values, as studied 10-12 months after implantation. Those authors also noted that D-1 receptor density in the SN was restored to normal at the time of study. Conceivably, differences in the length of time which has elapsed since
lesion and foetal graft surgery may affect the density of striatal (and possibly nigral) D-1 receptors, and may explain our differences.

As with D-2 receptors, the effect (or lack of it) of L-DOPA treatment on striatal D-1 receptors, may be influenced by the particular L-DOPA treatment regime adopted. Juncos et al (1989) found that D-1 receptor density increased in the 6-OHDA lesioned striatum of animals treated with continuous L-DOPA, but no change was observed in those treated intermittently. In combined autoradiographic and morphochemical studies, Ariano et al (1988, 1989, 1991) reported a dissociation between the D-1 receptor site and adenylate-cyclase positive neurones; this dissociation was partially restored by continuous and intermittent administration of L-DOPA. However, continuous administration of L-DOPA resulted in an increase in D-1 receptors in the lesioned striatum, whereas intermittent drug treatment resulted in a further down-regulation. In the present study, small reductions in D-1 binding were observed in the VL region of the lesioned striatum at Level 2 which were not reversed by the foetal graft or this L-DOPA treatment regime.

Relevance of autoradiographic findings
The potential relevance of these findings to the pathophysiology of L-DOPA-related motor abnormalities in Parkinson's disease, and the possible amelioration of such problems by foetal grafts, awaits further clarification of the role of DA receptors in L-DOPA related dyskinesias. In particular, the possible contribution of the newly described DA receptors (Giros et al 1989; Monsma et al 1989; Dearry et al 1990; Sokoloff et al 1990; Q-Y Zhou et al 1990; Sunahara et al 1991; Van Tol et al 1991) identified by molecular biological techniques, will have to be addressed.
Chapter 6

EFFECTS OF LONG-TERM TREATMENT (27 WEEKS) WITH L-DOPA AND CARBIDOPA ON MOTOR FUNCTION, GRAFT SURVIVAL AND GLIOSIS IN 6-OHDA-LESIONED RATS RECEIVING FOETAL VENTRAL MESENCEPHALIC GRAFTS
6.1. INTRODUCTION

Earlier Chapters have described how treatment of 6-OHDA lesioned rats receiving foetal ventral mesencephalic (VM) grafts with L-DOPA and carbidopa for 5 weeks did not interfere with the functional effects of the grafts nor with dopamine (DA) cell survival and fibre outgrowth. However, it is possible that no adverse effects on graft survival and fibre outgrowth were seen because the period of treatment was too short. Conceivably, the implanted foetal DA cells may become susceptible to the toxic effects of L-DOPA metabolites as they age, if treated with L-DOPA for longer periods. In addition, DA cell survival and fibre outgrowth from the grafts were examined some weeks after stopping L-DOPA treatment. It is possible that DA cells in the grafts may have been injured by the drug treatment, but had recovered from earlier damage during the drug-free period. It is also possible that L-DOPA treatment may injure grafted cells but not sufficiently to cause cell death. Present techniques for studying sub-lethal cell damage are limited. However, one indicator of toxicity might be the presence of a reactive gliosis, especially if it was accompanied by a reduction in survival of grafted TH-positive cells.

For these reasons the effect of long term treatment with L-DOPA and carbidopa (a total of 27 weeks) on the functional effects and survival of foetal VM grafts was studied using drug-induced rotation tests and TH-immunohistochemistry. In addition, the effect of this treatment on the astrocytic response within and surrounding the grafts was studied using glial fibrillary acidic protein immunohistochemistry (GFAP-IHC).

6.2. METHODS

6.2.1. Animal subjects
Six animals from each of the large groups described in
Chapter 3 were selected for the long-term study (see also section 2.10). The treatment groups were as follows:

**Group A (n=6):** 6-OHDA lesion + sham graft  
**Group B (n=6):** 6-OHDA lesion + sham graft + L-DOPA and carbidopa  
**Group C (n=6):** 6-OHDA lesion + foetal graft  
**Group D (n=6):** 6-OHDA lesion + foetal graft + L-DOPA and carbidopa

At the time of selection, all animals had received a unilateral 6-OHDA lesion, a foetal VM graft (groups C and D) or sham graft (groups A and B), and 5 weeks' treatment with L-DOPA and carbidopa in the drinking water (for groups B and D). In addition, all animals had been tested for apomorphine and (+)-amphetamine induced rotation on 3 separate occasions: tests 1 and 2 were performed prior to foetal or sham graft surgery, and test 3 was performed 6 weeks (for (+)-amphetamine) or 8 weeks (for apomorphine) after such surgery. Animals in groups A and B were taken randomly from the corresponding larger groups described in Chapter 3, the only requirement being a continued demonstration of high rates of contralateral rotation to apomorphine and ipsilateral rotation to (+)-amphetamine during the behavioural tests. Animals in groups C and D were selected on the basis of having shown a reduction in (+)-amphetamine-induced ipsilateral rotation of at least 85%, when tested 6 weeks after foetal graft surgery (test 3, see section 3.3.3). All groups were studied for a further 23 weeks, during which period groups B and D received a further 22 weeks' treatment with L-DOPA and carbidopa.

### 6.2.2. Behavioural assessment

**Apomorphine-induced rotation**  
Apomorphine-induced rotation tests 1, 2 and 3 were performed using a dose of 0.5mg/kg sc (see Chapter 3).
However, because the reduction in contralateral rotation in animals with foetal grafts in test 3 was small (although still significant), subsequent testing (test 4) of the animals in this long-term experiment was carried out with a lower dose of apomorphine (0.05mg/kg sc). Previous studies have shown that a lower dose of apomorphine may reveal the extent of behavioural recovery produced by a foetal graft more readily than a higher dose (Bjorklund et al 1980a; Dunnett et al 1981a). Test 4 was performed approximately 30 weeks after foetal or sham graft surgery, and 3-4 days after stopping L-DOPA and carbidopa treatment (in groups B and D).

(+)-Amphetamine-induced rotation. All (+)-amphetamine-induced rotation tests were performed with a dose of 5mg/kg ip. Test 4 was carried out approximately 30 weeks after foetal or sham graft surgery, and 3-4 days after stopping L-DOPA and carbidopa treatment (in groups B and D).

6.2.3. L-DOPA and carbidopa treatment
Animals in groups B and D had already received 5 weeks' treatment with L-DOPA and carbidopa, commencing 18-24 hours after foetal or sham graft surgery. After a drug-free period of approximately 3 weeks (during which time the third apomorphine and (+)-amphetamine-induced rotation tests were performed) drug treatment was recommenced in these animals. L-DOPA and carbidopa treatment was continued for a further 22 weeks, giving a total of 27 weeks treatment. L-DOPA and carbidopa were administered in the drinking water at target doses of 200mg/kg/24h and 25mg/kg/24h respectively, to animals housed in groups of 6, as described in section 2.5.

The time course of surgical procedures, behavioural testing and drug treatment is shown in Figure 6.1.
6.2.4. Immunohistochemistry (see section 2.6.4.)
Approximately 30 weeks after sham graft (groups A and B) or foetal graft (groups C and D) surgery (and approximately 3 days after discontinuing L-DOPA and carbidopa in groups B and D), animals were anaesthetised with pentobarbital (50mg/kg i.p.). Brains were fixed by perfusion with 4% paraformaldehyde in 0.1M PBS. Coronal sections 20μm thick were cut on a cryostat (Bright Instruments) at -20° C, through the entire striatum and SN into PBS. Three parallel series of sections were taken for TH and GFAP immunostaining and cresyl violet staining.

6.2.5. Cell counting
For the SN and VTA, TH-positive cells were counted separately on the intact and lesioned sides. Three sections (20μm) taken at the level of A -5.8 from bregma (Paxinos and Watson 1982) were selected from each animal and mean cell numbers determined. For the striatum every third section (20μm) was examined for TH-positive fibres and cell bodies within the grafts. The total number of cells in the grafts and the graft volumes were estimated as described in section 2.6.5.1. TH-positive cell size in the foetal grafts was assessed by measuring the long and short axes of 10 adjacent cells in the dorsal part of each graft. A mean value of cell size in the grafts was then calculated for each animal.

A semi-quantitative estimate of the density of GFAP positive cells in 20μm sections through the striatum was made as described in section 2.6.5.2.

6.2.6. Statistical analysis
(+) Amphetamine-induced rotation was compared within each group with that obtained in the immediately preceding test using a paired Student's t-test. Apomorphine-induced contralateral rotation obtained in test 4 could not be compared with rotation rates obtained in earlier tests because of the difference in doses. Therefore, the
rotation rates obtained in test 4 were compared in isolation using a 2-way ANOVA. Inter-group comparisons of (+)-amphetamine- and apomorphine-induced rotations at each test were made using a 2-way ANOVA and post hoc Dunnett's test.

For body weights, a 2-way ANOVA was used to assess the effect of treatment with L-DOPA and carbidopa and of foetal DA cell grafts in the 4 groups.

For the TH histological data, cell counts in the SN and VTA of the intact and lesioned sides were compared within each group using a paired Student's t-test. Cell numbers in the SN and VTA of the different groups were compared using a 2-way ANOVA. Total TH-positive cell numbers and cell sizes within the grafts and graft volumes in groups C and D were compared using Student's independent t-test. Due to the large variation in TH-positive cell numbers in the grafts of animals even within the same group, a square root transformation was also used to compare these two groups.

For the GFA-P histological data, cell numbers on the intact and lesioned/sham- or foetal-grafted striata were compared within each group for each of the 3 positions, and for the striatum as a whole, using mean values in a paired Student's t-test. A 2-way ANOVA was used to compare the cell density in the 4 groups. Any correlation between the glial cell density and TH-cell number within the grafts, and graft volume were assessed using a least squares linear regression test.

6.3. RESULTS

6.3.1. Fluid intake and body weight
Administration of L-DOPA and carbidopa at target doses of 200 mg/kg/24h and 25 mg/kg/24h respectively, caused a decrease in the volume of fluid drunk by animals in groups
Figure 6.1. Time course of surgical procedures, L-DOPA treatment and behavioural testing.

The time course of events in the weeks following unilateral 6-OHDA lesion surgery is shown. APO and Amph refer to the apomorphine and (+)-amphetamine tests for drug-induced rotation which were carried out. All (+)-amphetamine tests were performed with 5mg/kg ip and rotation was monitored over 1 hour immediately following injection. The first 3 apomorphine tests were performed with 0.5mg/kg sc apomorphine; the dose used in the final test was 0.05mg/kg sc. Rotations were monitored over 30 minutes immediately following injection of apomorphine. Sham or foetal graft surgery was performed soon after the second apomorphine- and (+)-amphetamine-induced rotation tests. The horizontal bars show the periods of treatment with L-DOPA and carbidopa of animals in groups B and D. In each case, behavioural tests on these animals were performed after a drug-free period of at least 3 days.
B (receiving sham grafts) and D (receiving foetal grafts), from a mean of 18.0 mls/rat/24h in group B and 19.7 mls/rat/24 h in group D prior to drug administration to means of 10.2 and 10.7 mls/rat/24 h respectively (p<0.001 both groups) after commencing drug treatment (Figure 6.2).

Mean doses of the drugs received over the 22 weeks of treatment were 185 mg±9.6/kg/24h (L-DOPA) and 23.1 +1.1mg/kg/24h (carbidopa) for group B, and 196 +12.5mg/kg/24h (L-DOPA) and 24.1 +1.5mg/kg/24h (carbidopa) for group D. When L-DOPA and carbidopa treatment was discontinued at the end of the experiment, fluid intake in groups B and D increased within 24 hours to levels similar to those found in groups A and C.

Administration of L-DOPA and carbidopa in groups B and D over the 22 week period was associated with an initial loss of body weight compared with groups A and C, after which body weight remained relatively stable (Figure 6.3). The decrease in body weight associated with L-DOPA and carbidopa treatment was significant (F(1,20)=7.9, p=0.01; ANOVA). The presence of a foetal graft was without effect, and there was no interaction between the effect of drug treatment and foetal graft on body weight. When drug treatment was discontinued there was an increase in body weight beginning within 24 hours.

Rats with a 6-OHDA lesion receiving L-DOPA and carbidopa showed intermittent spontaneous contralateral rotation during drug treatment, which was most marked when the animals were handled. In addition, they showed piloerection and increased grooming.

6.3.2. Rotational behaviour

(+)-Amphetamine-induced rotation (Figure 6.4A and B)
Rats in groups A and B showed marked ipsilateral rotation following administration of (+)-amphetamine, when tested
Figure 6.2. Mean fluid intake before, during and after L-DOPA treatment period.

The mean fluid intake of each group of rats is shown immediately before, during and immediately after the 22 week period over which groups B and D were treated with L-DOPA and carbidopa. All animals had received a 6-OHDA lesion. Rats in groups A (—□—) and B (—■—) received sham grafts whilst those in groups C (—○—) and D (—●—) received foetal VM grafts. The horizontal bar represents the period of L-DOPA treatment. L-DOPA and carbidopa treatment was associated with a reduction in fluid intake, which persisted for the duration of treatment. As individual intakes were not assessed SEM values are not available.

* indicates p<0.01 compared with pre-treatment intake, student's t-test.
Figure 6.3. Mean body weights before, during and after the L-DOPA treatment period.

The mean body weight of each group of rats is shown immediately before, during and immediately after the 22 week period over which groups B and D were treated with L-DOPA and carbidopa. All animals had received a unilateral 6-OHDA lesion. Animals in groups A (—□—) and B (—■—) received sham grafts and those in groups C (—○—) and D (—●—) received foetal grafts. The horizontal bar represents the period of L-DOPA treatment. L-DOPA and carbidopa was associated with a reduction in body weight which persisted for the duration of treatment.
prior to sham graft surgery (tests 1 and 2), and at 6
weeks (test 3) and 30 weeks (test 4) after sham graft
surgery. Rotation scores increased between tests 1 and 2
in both groups (t=2.6, df=5, p<0.05 for group A; t=3.07,
df=5, p<0.05 for group B). There was no further change in
rotation rates in subsequent tests for group A (sham graft
only). For group B (sham graft and L-DOPA and carbidopa
treatment) there was no change in rotation rates after 5
weeks' treatment with L-DOPA and carbidopa (test 3
compared with test 2), but there was a small reduction in
rotation after the second, 22 weeks period of treatment
(test 4 compared with test 3, t=3.14, df=5, p<0.05).

Animals receiving a unilateral 6-OHDA lesion followed by a
foetal graft alone (group C) also showed an increase in
ipsilateral rotation between tests 1 and 2, both of which
preceded foetal graft surgery (t=2.64, df=5, p=0.046).
After foetal graft surgery, animals in group C showed
almost complete abolition of (+)-amphetamine-induced
ipsilateral rotation at 6 weeks (test 3 compared with test
2; t=8.01, df=5, p<0.001), with all except one animal
showing a net mean contralateral rotation. This effect
was still present when the animals were tested 30 weeks
after foetal graft surgery (test 4 compared with test 2;
t=6.24, df=5, p<0.001), at which point all animals showed
a net mean contralateral rotation. The mean rate of
rotation was not significantly different at 6 weeks after
foetal graft surgery and at 30 weeks.

Animals in group D (unilateral 6-OHDA lesion followed by a
foetal graft and L-DOPA and carbidopa treatment) showed a
small, non-significant increase in ipsilateral rotation
between tests 1 and 2, performed prior to foetal graft
surgery. After foetal graft surgery, these animals showed
almost complete abolition of (+)-amphetamine-induced
ipsilateral rotation when tested at 6 weeks, having
received 5 weeks' treatment with L-DOPA and carbidopa,
(test 3 compared with test 2: t=7.46, df=5, p<0.001),

211
with all except two animals showing a net mean contralateral rotation. When tested 30 weeks after foetal graft surgery, having received a total of 27 weeks' treatment with L-DOPA and carbidopa, all animals in this group showed a net mean contralateral rotation, which was significantly increased compared with that obtained at 6 weeks (test 4 compared with test 3: *t*=4.78, df=5, *p*<0.05).

Mean (+)-amphetamine-induced rotation rates in the 4 groups were compared separately at 6 weeks (test 3) and at 30 weeks (test 4) after foetal graft or sham graft surgery (ANOVA). There was no effect of treatment with L-DOPA and carbidopa alone on behavioural recovery at either 6 or 30 weeks. The foetal graft produced a marked reduction in ipsilateral rotation at both time points (*F*(1,19)=175.5, *p*<0.001 at 6 weeks; *p*<0.01 *post hoc* Dunnett's test; and *F*(1,19)=179.7 *p*<0.001 at 30 weeks; *p*<0.01 *post hoc* Dunnett's test), with no interaction between the L-DOPA and carbidopa treatment and the foetal graft on rotation rates at either time point.

Following a 6-OHDA lesion but before implantation of a foetal or sham graft, (+)-amphetamine-induced ipsilateral rotation peaked at approximately 18 minutes following injection (Figure 6.2B). High rates of rotation then persisted for the rest of the 1 hour test period. Following graft surgery (test 3), the pattern of rotation changed in rats receiving foetal grafts. Many animals rotated contralaterally at high rates 6-10 minutes after injection. Rotation rates then rapidly declined, so that by 24 minutes after injection some rats were not rotating at all. The peak contralateral rotation score (at 6-10 minutes) was higher in animals receiving foetal grafts only (group C) than in animals receiving foetal grafts plus L-DOPA and carbidopa treatment (group D), but the difference was not significant). This pattern was still evident in both groups C and D in the final test.
Figure 6.4A. Mean (+)-amphetamine-induced rotation at various points during the study period in all groups.

Mean rotation rates observed over a 1 hour period immediately following injection of (+)-amphetamine (5mg/kg ip) are shown at various times over the experimental period. All animals received a unilateral 6-OHDA lesion. Animals in groups A (—□—) and B (—■—) received sham grafts. Animals in groups C (—○—) and D (—●—) received foetal VM grafts. Rats in groups B and D received chronic treatment with L-DOPA and carbidopa. The horizontal bar represents the period of L-DOPA treatment. Animals receiving sham grafts (groups A and B) continued to show marked ipsilateral rotation in response to (+)-amphetamine (5mg/kg ip) at each test. Animals receiving foetal grafts (groups C and D) showed marked reduction in ipsilateral rotation when tested at 6 and 30 weeks after implantation.

* indicates p<0.001 compared with pre-graft test.
+ indicates p>0.05 compared with test 3.
Figure 6.4B. Time course of (+)-amphetamine-induced rotation over 1 hour test periods before and after sham- or foetal-graft surgery.

The time course of (+)-amphetamine induced rotation over 1 hour in all groups is shown prior to sham- or foetal graft surgery (solid lines); dashed lines show the time-course after sham graft surgery (groups A and B), foetal graft surgery (groups C and D) and 27 weeks treatment with L-DOPA and carbidopa (groups B and D). group A (— ), group B ( — ), group C ( — ), group D ( — ). Positive values are rotations ipsilateral to the lesion/graft; negative values are contralateral. Values are the mean for each group of rats over the 1 hour period.
Apomorphine-induced rotation (Table 6.1)
In all groups of animals, there was no change in contralateral rotation rates following administration of apomorphine (0.5mg/kg) between the first and second tests (both performed prior to foetal or sham graft surgery and commencement of L-DOPA treatment).

For animals in group A, rotation rates remained unchanged after the sham graft (test 3 compared with test 2). High rates of contralateral rotation were also observed in test 4, using the lower dose of apomorphine (0.05mg/kg sc). For animals in group B, contralateral rotation rates increased after 5 weeks' treatment with L-DOPA and carbidopa (test 3 compared with test 2; \( t=3.78, \) \( df=5, \) \( p=0.013 \)). High rates of contralateral rotation were still observed in this group in test 4 using the lower dose of apomorphine (0.05mg/kg sc) after 27 weeks' treatment with L-DOPA.

Rats in group C showed a reduction in contralateral rotation rates when tested 8 weeks after foetal graft surgery (test 3 compared with test 2; \( t=2.6, \) \( df=5, \) \( p<0.05 \)), as did rats in group D (test 3 compared with test 2; \( t=4.22, \) \( df=5, \) \( p<0.01 \)). In the final test (test 4) performed 30 weeks after foetal graft surgery, using the lower dose of apomorphine (0.05mg/kg sc) contralateral rotation was markedly lower both in group C and in group D than in rats receiving sham grafts (groups A and B). Groups C (no L-DOPA treatment) and D (L-DOPA treatment for a total of 27 weeks) showed similar rotation rates.

Separate comparisons were made between the 4 groups of the mean scores obtained at 8 weeks (test 3, using apomorphine 0.5mg/kg sc) and at 30 weeks (test 4, using apomorphine 0.05mg/kg sc) using a 2-way ANOVA. For the analysis of test 3 results test 2 (apomorphine 0.5mg/kg sc) was used as covariate. L-DOPA treatment was associated with an increase in contralateral rotation rates at both tests.
Table 6.1. Apomorphine-induced contralateral rotation rates

<table>
<thead>
<tr>
<th>GROUP</th>
<th>PRE-FG/SG</th>
<th>POST-FG/SG</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEST</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>A (n=6) 6-OHDA lesion + SG</td>
<td>9.4±1.3</td>
<td>9.7±1.2</td>
</tr>
<tr>
<td>B (n=6) 6-OHDA lesion + SG + L-DOPA</td>
<td>13.3±1.5</td>
<td>12.5±1.5</td>
</tr>
<tr>
<td>C (n=6) 6-OHDA lesion + FG</td>
<td>10.0±1.1</td>
<td>11.5±1.3</td>
</tr>
<tr>
<td>D (n=6) 6-OHDA lesion + FG + L-DOPA</td>
<td>9.3±0.7</td>
<td>11.4±1.0</td>
</tr>
</tbody>
</table>

* indicates p<0.05 compared with test 2.

Mean (± 1 SEM) rotation rates observed over 30 minutes immediately following administration of apomorphine at various time points over the experimental period are shown. All animals received a unilateral 6-OHDA lesion. Animals in groups A and B received a sham graft (SG); animals in groups C and D received a foetal graft (FG). Rats in groups B and D received long-term treatment with L-DOPA and carbidopa. Test 1 was performed approximately 1 week after lesion surgery, and test 2 was performed just before FG or SG surgery. Test 3 was performed 8 weeks after FG or SG surgery, and after 5 weeks' treatment with L-DOPA and carbidopa (groups B and D). Apomorphine 0.5mg/kg sc was used for tests 1-3. Test 4 was performed approximately 30 weeks after SG or FG surgery, when animals in groups B and D had received a total of 27 weeks' treatment with L-DOPA and carbidopa. Apomorphine 0.05mg/kg sc was used for this test.
The foetal graft was associated with a reduction in contralateral rotation rates at both test points \( F(1,18)=48.2, p<0.0001 \) at 8 weeks; and \( F(1,18)=123.1, p<0.0001 \) at 30 weeks. There was an interaction between the foetal graft and L-DOPA treatment causing a reduction in apomorphine-induced contralateral rotation \( F(1,18)=10.11, p=0.005 \) at 8 weeks; and \( F(1,18)=25.3, p<0.001 \) at 30 weeks. The time course of rotation to apomorphine was unchanged by the foetal graft and by L-DOPA treatment.

### 6.3.3. Tyrosine hydroxylase immunohistochemistry

#### SN and VTA (Table 6.2)

The ventral mesencephalon \((A -5.8\) from bregma, Paxinos and Watson 1982) was studied in animals receiving foetal grafts \((n=5\) for group C, and \(n=6\) for group D) and in animals receiving sham grafts \((n=3\) in each of groups A and B). In all cases the 6-OHDA lesion resulted in a loss of >96% of the TH-positive cells on the lesioned SN compared with the intact side \((p<0.001\) all groups). The extent of cell loss in the VTA on the lesioned side was much more variable \((\text{range 21-46}\% \text{ of the intact side})\). There was no effect of either L-DOPA treatment \((\text{groups B and D})\) or the foetal graft \((\text{groups C and D})\) on the numbers of TH-positive cells in the intact SN and VTA of the host animals at the level studied \((\text{ANOVA})\). However, the L-DOPA treatment resulted in a further reduction in TH-positive cells found in the VTA on the lesioned side \((F(1,12)=6.23, p=0.02; p<0.05, \text{ post hoc Dunnett's test})\).

#### Intrastriatal foetal VM grafts

In animals receiving sham grafts \((\text{groups A and B})\) there were no TH-immunoreactive fibres or cells found in the lesioned striatum. In all animals receiving intrastriatal foetal VM grafts \((\text{groups C and D})\), TH-positive neurones were identified in the implanted striata. Grafts were
Table 6.2. TH-positive cell numbers in sections through the SN and VTA of the intact and lesioned VM.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Intact</th>
<th>Lesioned</th>
<th>% Intact</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SN</td>
<td>VTA</td>
<td>SN</td>
<td>VTA</td>
<td>SN</td>
</tr>
<tr>
<td>A (n=3):</td>
<td>147±18</td>
<td>57±9</td>
<td>3.8±2.1</td>
<td>25.6±5.4</td>
<td>2.4</td>
</tr>
<tr>
<td>6-OHDA lesion + SG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B (n=3):</td>
<td>129±9</td>
<td>53±12</td>
<td>3.8±2.1</td>
<td>*16.5±2.5</td>
<td>3.4</td>
</tr>
<tr>
<td>6-OHDA lesion + SG + L-DOPA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C (n=5):</td>
<td>130±10</td>
<td>88±18</td>
<td>2.4±2.3</td>
<td>35.0±5.0</td>
<td>2.5</td>
</tr>
<tr>
<td>6-OHDA lesion + PG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D (n=6):</td>
<td>135±7</td>
<td>76±10</td>
<td>3.3±1.6</td>
<td>*16.4±3.2</td>
<td>2.4</td>
</tr>
<tr>
<td>6-OHDA lesion + PG + L-DOPA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ±1 SEM TH-positive cell numbers found in 20µm sections from the SN and VTA taken through level -5.8 from bregma according to Paxinos and Watson (1982). All animals received unilateral 6-OHDA lesions. Animals in groups A and B received sham grafts, whilst those in groups C and D received foetal VM grafts. Animals in groups B and D received treatment with L-DOPA and carbidopa for a total of 27 weeks. In all animals there was a significant loss of TH-positive cells in the SN and VTA of the lesioned side compared with the intact side. Treatment with L-DOPA and carbidopa had no effect on TH-positive cell numbers remaining in the intact SN and VTA. However, this treatment resulted in a significant further reduction in the number of TH-positive cells found in the VTA on the lesioned side (*p=0.02, ANOVA)
found mainly in the dorsal and mid-striatum but some extended more ventrally. TH-positive cells were found clustering at the graft periphery and at the dorsal and ventral poles. TH-positive fibres could be seen extending for a short distance (<1mm) around the grafts. TH-positive fibres and cells found in an animal treated with L-DOPA and carbidopa for a total of 27 weeks are shown in Figure 6.5A and B.

A comparison was made between the number of TH-positive cells in the grafts of animals in group C (foetal graft alone) and group D (foetal graft plus long-term L-DOPA and carbidopa treatment). Where two grafts were identified in the striatum of any animal, cell numbers were combined to give a single total value. There was no difference in the numbers of TH-positive cells in the grafts of animals in group C (mean 1061±302.0, range 236-2088) and in group D (mean 924.6±284.5, range 162-2094), Student's t-test. The size of the TH-positive cells in the grafts also did not differ in the 2 groups (Long axis: group C mean=19.2±1.38; group D mean=18.91±0.73; Student's t-test. Short axis: group C mean=13.14±0.86; group D mean=12.73±1.07; Student's t-test). The combined volumes of the grafts in animals treated with L-DOPA and carbidopa (group D) ranged from 1.06-2.3mm³ (mean=1.50±0.62), and in animals receiving foetal grafts alone (group C) ranged from 0.69-3.1mm³ (mean=1.78±0.48), with no difference between the two groups (t=0.46, df=10, p=0.65).

6.3.4. GFAP-immunoreactivity (Table 6.3)
In all groups, GFAP-positive cells ('glial cells') in the cortex appeared quiescent (apart from in the needle tract, where a reactive gliosis was observed) on both sides of the brain. In both striata of animals from all groups, the density of glial cells was regionally variable. Glial cell density was greatest in the dorsal (position 1) and medial (position 3) areas of the striatum and lowest in the lateral striatum (position 2). In groups C and D
### Table 6.3. Glial cell density in intact and lesioned/grafted striatum

<table>
<thead>
<tr>
<th>Position</th>
<th>No</th>
<th>TOTAL</th>
<th>Group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTACT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>72.0±1.6</td>
<td>44.7±3.6</td>
<td>65.2±4.1</td>
<td>60.6±4.1</td>
<td>60.5±8.9</td>
<td>55.2±3.0</td>
<td>66.0±12.7</td>
</tr>
<tr>
<td>2</td>
<td>82.1±7.2</td>
<td>44.3±4.6</td>
<td>74.4±11.2</td>
<td>66.8±5.9</td>
<td>76.1±5.5</td>
<td>50.4±4.4</td>
<td>76.5±11.0</td>
</tr>
<tr>
<td>3</td>
<td>93.7±18.0</td>
<td>39.0±3.7</td>
<td>71.5±9.8</td>
<td>68.1±8.7</td>
<td>114.5±4.0</td>
<td>*72.5±11.0</td>
<td>91.5±3.9</td>
</tr>
<tr>
<td>TOTAL</td>
<td>258.8±28.5</td>
<td>168.7±28.5</td>
<td>195.8±21.5</td>
<td>165.6±21.5</td>
<td>187.6±21.5</td>
<td>135.5±21.5</td>
<td>188.0±21.5</td>
</tr>
</tbody>
</table>

* indicates p<0.05 or less compared with intact side.

Values are mean glial cell density ± 1 SEM per high power field for each of the 3 positions shown in the diagram. The Total value refers to the mean of all positions combined for each animal. A minimum of 5 sections (20um thickness) per animal taken through levels corresponding to foetal or sham graft placements were analysed. All animals received a unilateral 6-OHDA lesion. Animals in groups A (n=4) and B (n=4) received sham grafts, and animals in groups C (n=5) and D (n=6) received foetal grafts into the 6-OHDA lesioned striatum. Animals in groups B and D received treatment with L-DOPA and carbidopa for 27 weeks.
Figures 6.5A and B. TH-immunohistochemistry of a foetal VM graft in a rat treated with L-DOPA and carbidopa.

A low power view of the dorsal part of an intra-striatal foetal DA cell graft is shown from an animal treated for a total of 27 weeks with L-DOPA and carbidopa (6.5A). The area marked by arrow heads is shown at higher power in 6.5B. Many TH-positive cells were found, with some extending long fibres into the surrounding striatum.
glial cell density was especially high in the immediate vicinity of the foetal grafts. The mean glial cell density in the right (intact) and left (lesioned and sham or foetal grafted) striata is shown in Table 6.3.

In animals receiving sham grafts alone into the lesioned striatum (group A) or followed by long-term L-DOPA and carbidopa treatment (group B), there was no difference (within each group) between the glial density found in the intact striatum and that found in the sham-grafted striatum, when considered either as a mean total value or at any of the individual positions.

In animals receiving foetal grafts (groups C and D), the glial cells in the grafted striatum had thicker processes and were more intensely stained than those in the intact striatum (Figure 6.6). In animals receiving a foetal VM graft alone (group C), the mean density of GFAP-positive cells was higher in all positions in the grafted striatum compared with the intact side. However, the difference only reached significance in the lateral area (position 2) of the grafted striatum ($t=3.22, df=3, p<0.05$; Student's $t$-test), and when all areas were considered together ($t=3.38, df=3, p<0.05$). In rats receiving foetal grafts and L-DOPA and carbidopa treatment (group D), there was a significant increase in glial cell density in the dorsal and lateral areas (positions 1 and 2) and in the total lesioned and grafted striatum, when compared with the intact side ($p<0.01$). Using the mean total values for all positions, the glial density in the intact striatum was not affected by the presence of a foetal graft in the opposite hemisphere nor by treatment with L-DOPA and carbidopa and there was no interaction (ANOVA). In contrast, in the implanted striatum a foetal graft (groups C and D) was associated with a marked increase in glial cell density in the surrounding striatum ($F(1,15)=18.99, p<0.001$, ANOVA, with intact side as covariate), which was unaffected by L-DOPA and carbidopa treatment. There was no
interaction between foetal graft and L-DOPA treatment on glial density.

The grafts in animals from groups C and D were clearly demarcated from the surrounding host striatum by a rim of glial cells and fibres (Figure 6.7A). The thickness of the rim tended to be greatest at the dorsal and ventral poles. Glial cells were also found in the central areas of the grafts. Glial cell processes were commonly associated with blood vessels in or adjacent to the graft (Figure 6.7B). In some grafts (especially those of smaller size) glial cells were found throughout the graft, without forming a discernible rim.

In both groups of animals receiving foetal grafts, the glial cell density within the grafts varied widely. In group C, the mean density within the grafts was less than that in the adjacent host striatum (73.2±8.5 per high power field compared with 93.2±4.8; t=3.97, df=4, p=0.029; Student's t-test) although the cells within the graft were more intensely stained. However, in animals that had received treatment with L-DOPA and carbidopa for a total of 27 weeks (group D), the mean glial cell density was higher (though not significantly) within the grafts than the surrounding striatum (139±17.5 compared with 101.1±5.3; t=2.35, df=5, p=0.07; Student's t-test). The mean glial cell density within the grafts of animals receiving L-DOPA and carbidopa (group D, mean 139±17.5 per high power field) was almost double that found in animals not receiving these drugs (group C, mean=73.2±6.9 per high power field); t=3.09, df=10, p<0.01 (Figure 6.7C and D).

However, there were some grafts in both groups of animals with very dense networks of GFAP-positive processes within the central area, so that counting of glial cells was difficult. There was no correlation between mean glial density within the grafts and the total number of TH cells (r=0.29), or with the graft volume (r=0.177).
Figure 6.6. GFAP-immunohistochemistry of the right and left striata of a rat receiving a foetal graft (graft not shown).

The glial reaction in the intact (right panel) and corresponding position of the lesioned/grafted striatum (left panel) of an animal receiving a unilateral 6-OHDA lesion 38 weeks earlier, followed by a foetal VM graft 30 weeks earlier, is shown. The left panel shows an area of implanted striatum remote from the graft. There was a marked increase in the density of glial cells in the grafted striatum and the cells appeared to be in a reactive state, compared with the intact side.
Figure 6.7. GFAP-immunostaining of the striatum containing a foetal VM graft in an animal not treated with L-DOPA (A-C) and in an animal receiving long-term L-DOPA treatment (D).

A. This photomicrograph shows the glial response in the area containing the foetal graft. Note the 'rim' of glial cells arranged around the periphery of the graft. In addition, the glial reaction in the striatum immediately surrounding the graft can be seen to be intense. Glial cells can also be seen within the central area of the graft.

B. Glial processes can be seen in close proximity to a blood vessel shown here at high power. This arrangement was a common finding both in immediate relation to the grafted area and also further afield in the host striatum.

C and D. The glial cell density found within grafts from an animal from group C (no L-DOPA) is shown in panel C and is significantly lower than that found within the grafts from an animal treated with L-DOPA and carbidopa (group D) shown in panel D.
6.4. DISCUSSION

The main findings of this study are: (1) The abolition of (+)-amphetamine-induced ipsilateral rotation produced by rat foetal VM grafts was fully developed 6 weeks after foetal graft surgery and persisted unchanged for many months. Apomorphine-induced contralateral rotation was greatly reduced 30 weeks after foetal graft surgery compared with rats receiving sham grafts. These effects of the grafts were not altered by chronic treatment with L-DOPA and carbidopa. (2) DA cells (as revealed by TH-immunostaining) in the foetal grafts survived for 30 weeks, and prolonged L-DOPA and carbidopa treatment did not affect this survival. (3) Foetal grafts produced a glial reaction within the implanted host striatum that was still evident 30 weeks after the foetal graft surgery. The glial reaction within the grafts was greater in animals treated with L-DOPA and carbidopa.

L-DOPA and carbidopa regime
The treatment regime used in this study was associated with a reduction in fluid intake and body weight which persisted for the duration of treatment. Possible reasons for these findings were discussed in section 3.3.2.

Drug-induced rotational behaviour

(i) (+)-Amphetamine
In group A ipsilateral rotation rates remained stable over the whole experimental period, demonstrating the robustness of the lesion for up to 38 weeks. Animals in group B had also shown stable rotation rates between tests 2 and 3, after receiving L-DOPA and carbidopa for 5 weeks (i.e. before the commencement of this study). However, these animals showed a reduction in ipsilateral rotation rates after treatment with L-DOPA and carbidopa for a further period of 22 weeks (i.e. test 4 compared with test 3). This observation contrasts with the enhanced
contralateral rotation that was observed in these animals after administration of apomorphine. Conceivably L-DOPA treatment may have different effects on postsynaptic receptors in the lesioned and intact striata. Thus, it is possible that L-DOPA exacerbates DA receptor supersensitivity in the lesioned striatum (so accounting for the increased contralateral rotation rates observed in these animals following administration of apomorphine) but results in a reduction of DA receptor sensitivity in the intact striatum (so accounting for the reduced ipsilateral rotation rates observed in these animals following administration of (+)-amphetamine).

Animals receiving a unilateral 6-OHDA lesion followed by a foetal graft either alone (group C) or with L-DOPA treatment (group D) had shown a net mean contralateral rotation when tested 6 weeks after graft surgery (i.e. test 3, performed prior to selection for this study), and continued to do so when tested 30 weeks after foetal graft surgery (test 4). There was no difference at either test between the groups, indicating that L-DOPA had not interfered with the behavioural recovery caused by the grafts. For animals receiving a foetal graft alone (group C), there was no difference between the rotation rates observed at 6 and 30 weeks, suggesting that the behavioural recovery caused by the graft was already fully developed by 6 weeks. However, for animals receiving a foetal graft followed by L-DOPA treatment (group D), there was a further increase in (+)-amphetamine-induced contralateral rotation rates between test 3 (performed 6 weeks after foetal graft surgery, and after 5 weeks' L-DOPA treatment), and test 4 (30 weeks after foetal graft surgery, and after a total of 27 weeks' treatment with L-DOPA). This finding again suggests that L-DOPA may exert different effects on the intact and lesioned (and grafted in this case) striatal DA receptors.
(ii) Apomorphine
The effectiveness of the 6-OHDA lesion was revealed by high rates of contralateral rotation following administration of apomorphine which persisted for 38 weeks after 6-OHDA lesion surgery in sham-grafted animals (groups A and B). The rotational rates were consistent with a >95% depletion of DA in the lesioned striatum (Ungerstedt 1971c, Hefti et al 1980). Rotation rates in animals receiving a unilateral 6-OHDA lesion followed by a sham graft (group A) were stable over time. Animals receiving a unilateral lesion followed by a sham graft and long-term treatment with L-DOPA and carbidopa (Group B), showed an increase in contralateral rotation rates after 5 weeks of treatment, compared with rats in group A. High rates of contralateral rotation were still found on the final apomorphine test, when a lower dose (0.05mg/kg) was used. Enhancement of DA agonist-induced behavioural supersensitivity by L-DOPA has been demonstrated previously both in un-lesioned (Klawans et al 1977, Bailey et al 1979) and lesioned animals (Parenti et al 1986), although one study found no change (Jackson et al 1983).

Animals receiving a unilateral 6-OHDA lesion followed by a foetal graft alone (group C) or with L-DOPA and carbidopa treatment (group D) had shown a small (but significant) reduction in apomorphine-induced contralateral rotation when tested 8 weeks after foetal graft surgery (test 3), prior to selection for this study. This differed from the persistent high rates of rotation found in the 2 control groups (A and B) receiving sham grafts. Use of a lower dose of apomorphine (0.05mg/kg sc) in the final test (test 4) revealed a marked reduction in contralateral rotation in animals with foetal grafts (group C and D) compared with those receiving sham grafts (groups A and B), as has been found by others (Bjorklund et al 1980b, Dunnett et al 1981a). This finding was especially noteworthy in animals receiving a foetal graft and long-term L-DOPA and carbidopa treatment (group D), since in the absence of a
foetal graft such treatment resulted (group B) in an increase in contralateral rotation compared with that found in animals with a 6-OHDA lesion alone (group A). The presence of a foetal graft in conjunction with L-DOPA and carbidopa treatment (group D) reduced not only the behavioural supersensitivity consequent upon the 6-OHDA lesion, but also the enhancement of such supersensitivity that would otherwise have resulted from the L-DOPA and carbidopa treatment.

**Tyrosine hydroxylase immunohistochemistry**

(i) **SN and VTA**
TH-positive cells in the intact SN and VTA did not appear to be affected by L-DOPA and carbidopa treatment for a total of 27 weeks. There has been no previous evidence that L-DOPA is harmful to DA cells in the SN or VTA of normal adult animals. Whether this drug is harmful to DA cells which are degenerating, however, is uncertain. The present results suggest that prolonged L-DOPA treatment may be harmful to cells which are degenerating, as this treatment was associated with a significant further reduction in number of TH-positive cells remaining in the VTA ipsilateral to the 6-OHDA lesion. This observation suggests that L-DOPA treatment may be harmful to cells previously exposed to a neurotoxin (in this case 6-OHDA), but not to cells which are basically healthy (such as those in the contralateral intact SN and VTA, and those found in the foetal grafts). In the results reported in Chapter 4 (where animals were treated with L-DOPA and carbidopa for 5 weeks), a reduction in TH-positive cell numbers in the VTA ipsilateral to the 6-OHDA lesion was also observed in L-DOPA treated animals, but less marked than that seen here. This difference may be due to the shorter period of treatment in the experiment described in Chapter 4, the fact that the animals used in that experiment were killed at a younger age and/or the fact that these animals had also had a drug-free period of 4-5
weeks prior to killing. This finding has obvious implications for the treatment with L-DOPA of patients with Parkinson's disease, in whom DA cells remaining in the SN and VTA may be degenerating (Yahr et al 1972).

(ii) Intrastriatal foetal VM grafts
The arrangement of TH-positive cells within the grafts of rats in groups C (foetal graft only) and D (foetal graft followed by prolonged L-DOPA and carbidopa treatment) were similar. The smallest number of TH-positive cells found in the grafts of any animal was 162, a number sufficient to result in significant behavioural recovery (Brundin et al 1985a, 1988a). There was no evidence of an inhibitory effect of L-DOPA on cell numbers within the grafts, although the smallest graft was found in an animal receiving L-DOPA. The size of TH-positive cells was not affected by L-DOPA treatment. Confirming earlier work (Nishino et al 1990), the numbers of surviving TH-positive cells in these grafts, which were studied 38 weeks after implantation, were comparable to the numbers of surviving cells found at an earlier stage (Chapter 4).

Recently, Steece-Collier et al (1990) reported that chronic L-DOPA treatment of rats receiving a foetal VM graft did not reduce the numbers of TH-positive cells surviving in the grafts, but caused a patchy reduction in fibre outgrowth and a reduction in TH-positive cell size. There are several possible explanations for our differences. First, a different transplantation technique was used (implantation of solid pieces of tissue rather than cell suspensions). Second, these authors used a different L-DOPA treatment regime, involving injections of Sinemet (levodopa:carbidopa 10:1; doses 75 mg levodopa/kg twice daily ip for the first 2 weeks, and then 37.5 mg levodopa/kg twice daily ip for the final 2 weeks). This treatment may have resulted in higher peak DA concentrations in the striatum than with drugs administered in the drinking water.
Although one of the main findings of Steece-Collier et al (1990) was that L-DOPA treatment resulted in a patchy reduction in DA fibre outgrowth from the grafts, formal quantification was not undertaken. Also, their statistical analysis of the cell size data may be unsound. Thus, two groups of rats (n=6 each) were transplanted, and one group was treated with Sinemet. Cell dimensions were measured in 10 grafted cells within each rat; a t-test was performed using each measured cell as an individual case number, yielding a total 'n' of 60 for each group. Clearly, the analysis of cell size should have been performed using a mean value for cell size for each animal (obtained by taking the mean value of the 10 cells studied for each rat), and an unpaired t-test performed on the two groups using an 'n' of 6 (and not 60) for each group. Whether there would still be a significant difference in cell size between the two groups if the correct analysis were used is unknown.

Steece-Collier et al (1990) also studied the effect of L-DOPA on monolayer cultures of foetal DA neurones, and found a reduction in TH-positive neurite outgrowth which was dose-dependent. Some fibre stunting was reported at L-DOPA concentrations in the culture fluid which were lower than those found in the plasma of patients receiving L-DOPA (Hare et al 1973). However, the relevance of the toxic effects of L-DOPA on DA cell cultures in vitro is uncertain. Thus, DA cell survival depends critically on the local environment, in particular on the presence of appropriate local support cells (O'Malley et al 1991), which may be absent in vitro.

**Glial reaction**

Glial density in the lesioned striatum of animals receiving sham grafts alone (group A) or followed by long-term treatment with L-DOPA and carbidopa (group B) did not differ from that found on the intact side. This observation indicates that a striatal needle wound
together with striatal DA nerve terminal degeneration is not associated with a permanent gliosis in the striatum, confirming previous findings (Bignami and Dahl 1976, Stromberg et al 1986, Abrous et al 1988, Ogawa et al 1989). Thus, by 38 weeks after the lesion and 30 weeks after the sham-graft, any glial reaction that may have been present at earlier times as a result of the surgical wound and/or nerve terminal degeneration or the surgical wound has subsided.

The presence of a foetal VM graft in the lesioned striatum of animals in groups C and D was associated with a gliosis in the surrounding striatum when examined 30 weeks after the implantation of the tissue. The increased glial density was no more widespread in the implanted striatum of animals receiving a foetal graft and treatment with L-DOPA (group D) than in those receiving foetal grafts alone (group C), and the intensity of staining appeared similar. Several studies have shown a gliosis following implantation of foetal brain tissue into the CNS (Azmitia and Whitaker 1983, Jaeger 1985, Abrous et al 1988, Krum and Rosenstein 1989, Zhou et al 1989), which can persist for up to 6 months, (Azmitia and Whitaker 1983) as found in this study. Abrous et al (1988) found an increased glial density in the 6-OHDA-lesioned striatum surrounding foetal VM grafts, which persisted for up to 4 weeks. As found by many of these authors, the grafts observed in this study were surrounded by a 'rim' of GFAP-positive cells whose processes were often associated with blood vessels. There was no evidence that this rim prevented outgrowth of TH-positive fibres from the graft.

In animals not treated with L-DOPA and carbidopa (group C), glial density within the grafts was lower than that in the surrounding striatum, although the cells appeared more densely stained. In contrast glial density in the grafts of animals receiving L-DOPA and carbidopa (group D) was slightly higher than that in the surrounding striatum
(though not significantly) and significantly higher than that found in the grafts of animals from group C. The importance of the glial reaction within the grafts and its exacerbation by concurrent treatment with L-DOPA and carbidopa is uncertain. As the same dose of L-DOPA (200mg/kg/24h) was administered to all animals in group D in this study, no conclusions can be drawn as to whether this effect is dose-related. Furthermore, the effect of duration of treatment is unclear, as glial density was not assessed after the same dose of L-DOPA was administered for the shorter period of 5 weeks (Chapter 4). However, there was no evidence that a greater glial reaction within the grafts was associated with a reduction in number of surviving TH-positive cells in the grafts or with an alteration of their morphology or a reduction of fibre outgrowth. A recent report indicates that reactive astrocytes, far from hindering axon outgrowth, may be permissive for their growth (David et al 1990). Shetty et al (1991) studied the long-term morphology of foetal nigral grafts in the anterior chamber of the eye and noticed a steady increase in the glial population as the transplants aged, but without an associated neuronal loss. As in the present study, Shetty et al reported that many of the glia possessed thick processes, which they suggest might be a compensatory response to ageing of the grafts.
Chapter 7

GENERAL DISCUSSION
7.1. SUMMARY OF FINDINGS

The aim of this thesis was to assess the effect of oral treatment with L-DOPA and carbidopa on the survival of rat foetal VM grafts, and on some of their functional effects, using a rat model of Parkinson's disease. The main findings can be summarised as follows:

1. Treatment of rats receiving a unilateral 6-OHDA lesion and rat foetal VM grafts with L-DOPA and carbidopa for 5 weeks did not interfere with the functional effects of the grafts. These effects consisted of a reduction of apomorphine-induced contralateral rotation, and abolition of (+)-amphetamine-induced ipsilateral rotation.

2. Treatment with L-DOPA and carbidopa for 5 weeks of 6-OHDA-lesioned rats receiving foetal grafts did not impair the survival of foetal dopamine cells within the grafts, as revealed by TH-immunohistochemistry. This treatment did not impair fibre outgrowth from the grafted dopamine cells, as revealed by $^3$H-mazindol autoradiography.

3. Foetal dopamine cell grafts restored the density of D-2 dopamine receptors in the lesioned striatum to values found in the control striatum (demonstrated with $^3$H-spiperone autoradiography), but did not affect the lesion-induced changes in D-1 receptor density (demonstrated with $^3$H-SCH 23390 autoradiography). L-DOPA treatment did not affect the density of D-1 or D-2 dopamine receptors. As mentioned in Chapter 5, the recent discovery by molecular biological techniques of additional dopamine receptor types may affect the interpretation of binding studies in the future.

4. Treatment of 6-OHDA-lesioned rats receiving foetal dopamine cell grafts with L-DOPA and carbidopa for a total of 27 weeks did not interfere with the functional effects of the grafts. TH-positive cell survival in
foetal grafts was not reduced by such long-term L-DOPA treatment. However, an increased glial reaction was observed within the grafts of animals treated with L-DOPA and carbidopa for 27 weeks.

5. A number of additional effects of L-DOPA treatment were observed in the course of this work, which are incidental to the issues concerning foetal grafts. These effects included:
- a reduction in fluid intake and body weight in L-DOPA-treated animals;
- an increase in apomorphine-induced stereotypy and rotation (both of which were reduced by foetal grafts);
- an increase in the density of $^3$H-mazindol binding in the intact striatum;
- a reduction in the number of TH-positive cells remaining in the lesioned VTA in animals treated with L-DOPA and carbidopa for 27 weeks.

In conclusion, the results presented in this thesis suggest that chronic L-DOPA treatment does not damage healthy foetal rat dopamine cells; or, if it does, the effect is slow and not sufficient to affect the survival or functional effects of grafts as studied in this model. However, long-term L-DOPA treatment may have some adverse effects on the foetal grafts, as revealed by the increased gliosis seen in grafts of treated animals.

An important question that arises at the end of this study is the relevance of these findings to the treatment with L-DOPA of patients with Parkinson's disease who receive human foetal DA cell grafts. There are some potentially important differences between the experimental protocols used in this study and the situation that exists in man, and there are several questions about the effects of L-DOPA which remain unanswered.
7.1. SUITABILITY OF THE 6-OHDA LESION MODEL OF PARKINSON'S DISEASE FOR THE STUDY OF FOETAL GRAFTS

7.1.1. Mechanism of cell damage
A major difference between the experimental model used for this study and the situation found in man is that the process(es) causing Parkinson's disease are not replicated. In patients with Parkinson's disease, the disease process, either alone or in conjunction with L-DOPA treatment, may be detrimental to graft survival or function. A unilateral 6-OHDA lesion produces dopamine cell damage which is finite, and the molecular mechanisms may be different from those occurring in Parkinson's disease. It is possible that Parkinson's disease is caused by a combination of exposure to one or more environmental or endogenous neurotoxins and a genetic susceptibility (Barbeau et al 1984), resulting in a slow loss of neurones over a life-time. The effect of such a process together with the effect of L-DOPA treatment on the implanted cells can not be assessed with the 6-OHDA model.

Nevertheless, there may be some similarity between the molecular mechanisms involved in 6-OHDA and L-DOPA-induced toxicity in vitro, and those believed to occur in Parkinson's disease. In Parkinson's disease, there is evidence of accelerated free radical production, which is an important mechanism of cell death produced by both 6-OHDA and L-DOPA metabolism, and increased lipid peroxidation (Dexter et al 1986, 1989). Furthermore, L-DOPA and DA can cause death of cultured dorsal root ganglion cells in the presence of ferric iron via a process of free radical generation and increased lipid peroxidation (Tanaka et al 1991). This finding may be of particular relevance to patients with Parkinson's disease receiving L-DOPA, in whom the SN contains elevated levels of iron and lipid peroxides (Dexter et al 1989). L-DOPA treatment when combined with an on-going disease
process, such as that occurring in Parkinson's disease, might prove to be toxic to dopamine cells in man. Indeed, the results presented in Chapter 6 suggest that adult DA cells previously exposed to a neurotoxin (such as those remaining in the VTA ipsilateral to the 6-OHDA lesion) might be susceptible to toxic effects of L-DOPA. In contrast, healthy cells (such as those in the contralateral SN and VTA, and in the foetal grafts) are not. In patients with Parkinson's disease, many of the DA cells remaining in the SN may be degenerating, as revealed morphologically (Yahr et al 1972) and by the fact that they contain reduced levels of TH and TH mRNA (McGeer and McGeer 1976; Javoy-Agid et al 1990). These degenerating cells may, like the VTA cells previously exposed to 6-OHDA studied in Chapter 6, be further harmed by treatment with L-DOPA. However, even if L-DOPA proved to be toxic to degenerating DA cells in the SN of patients with Parkinson's disease, healthy foetal DA cells, placed 'ectopically' into the striatum, might be protected from such an effect.

7.1.2. Pattern of dopamine cell loss
A further difference between the 6-OHDA model and Parkinson's disease is that the pattern and extent of dopamine cell loss in the midbrain is not identical. In this study a 6-OHDA lesion consistently produced complete degeneration of DA cells in the ipsilateral SN, whilst DA cell loss in the ipsilateral VTA was less marked and more variable. In Parkinson's disease, although DA cell loss is also much more variable in the VTA than in the SN, cell loss in the SN may be incomplete, with greatest losses in the caudal and ventrolateral parts (Bernheimer et al 1973). The pattern of DA cell loss in the SN in man affects the pattern of DA innervation in the striatum, which may further affect the extent to which implanted DA cells survive, and the extent to which toxic effects of L-DOPA may be manifested. The human situation might be more closely replicated experimentally if foetal
VM grafts were implanted into the striatum of animals with partial 6-OHDA lesions, and the effect of L-DOPA assessed in this situation. It has been found that the presence of an intact intrinsic septohippocampal innervation, survival and growth of cholinergic and sympathetic neurones implanted into the hippocampus is limited (Bjorklund and Stenevi 1981; Gage and Bjorklund 1986). However, recent studies suggest that an intact host nigrostriatal pathway does not inhibit the survival of dopamine cells implanted into the striatum (Schmidt et al 1981; Dunnett et al 1988; Doucet et al 1990), although fibre outgrowth may be reduced (Doucet et al 1990).

Further factors which might affect graft survival are the ages of the host and of the lesion. Studies of DA cell grafts in old animals, with recent lesions, suggest that host age is a relatively unimportant determinant of graft survival (Gage et al 1983). However, the survival of grafts in old rats with a long-standing lesion has not been assessed. This situation may be more relevant to neural grafting in Parkinson's disease.

7.1.3. Assessment of functional activity of grafts
In addition to whole-body motor asymmetry induced by drugs, a 6-OHDA lesion results in lateralised motor, sensorimotor and learning impairments. Thus, the precision, speed and coordination of use of the contralateral limb is impaired and the animals show a sensory neglect on the side of the body contralateral to the lesion, as well as defects in spatial orientation (Marshall and Teitelbaum 1974; Ljungberg and Ungerstedt 1976). Bilateral lesions also result in aphagia, adipsia and akinesia (Ungerstedt 1971d). Many of these different behavioural functions have been ascribed to different regions of the rat striatum, and their amelioration by foetal DA cell grafts is dependent on the exact placement of a graft (Dunnett et al 1983). In this study, functional activity of the grafts was assessed by
The 6-OHDA rat model is widely used for monitoring DA and DA receptor imbalance between the right and left striata. The induction of rotation by administration of apomorphine and (+)-amphetamine are established techniques for detecting both the completeness of a unilateral 6-OHDA lesion (Ungerstedt 1971b,c; Schmidt et al 1982, 1983), and for monitoring some of the behavioural effects of foetal VM grafts (Bjorklund and Stenevi 1980; Dunnett et al 1981, 1983, 1988). However, one limitation of using drug-induced rotation alone as an index of graft viability is that spontaneous activity of the grafts is not assessed directly. It is the spontaneous activity of foetal grafts which is important in man, and not their effects on drug-induced rotation. Apomorphine-induced rotation may depend on the sensitivity of dopamine receptors in the deafferented striatum, which in turn may reflect levels of chronic spontaneous dopamine release in the striatum. Reduction of apomorphine-induced contralateral rotation in rats with foetal grafts may therefore suggest that the grafts are spontaneously active. In the present study reduction of apomorphine-induced contralateral rotation was observed in foetal grafted rats, and that reduction was not adversely affected by L-DOPA treatment - possibly indicating that the spontaneous activity of the grafts was not hampered by L-DOPA treatment. However, a better index of spontaneous dopamine release by the grafts might have been obtained by using in vivo dialysis in conscious, freely moving animals.

In man, it is also important to know how the functional activity of grafts (including DA release) are affected by concurrent treatment with L-DOPA. Administration of L-DOPA may interfere with the spontaneous function of grafted DA cells via stimulation of 'autoreceptors' which are believed to exist on the grafted nerve terminals.
(Strecker et al 1987). Normally, autoreceptor activation results in a temporary inhibition of DA synthesis and release (Roth 1984). It is conceivable that DA neurones located in the striatum may be excessively inhibited by the exogenous L-DOPA (and subsequently DA), especially if they lack the normal afferent stimulatory inputs that DA cells in the nigra receive. In the 6-OHDA lesion model, the effect of concurrent L-DOPA treatment on graft activity (as revealed by drug-induced rotation) would be difficult to assess, because L-DOPA itself causes contralateral rotation in rats with a 6-OHDA lesion. However, the main concern of the present study was the question of chronic toxicity of L-DOPA on grafted DA cells. Therefore, in all rotation tests performed in this study, it was decided to stop L-DOPA treatment prior to behavioural testing, so that the effects of the grafts alone (i.e. uncomplicated by the rotational effects of concurrent L-DOPA administration) on apomorphine- and (+)-amphetamine-induced rotation could be assessed. If L-DOPA had been continued during the rotation tests, it would have been impossible to separate out the changes in drug-induced rotation that were due to the grafts, and those due to administered L-DOPA.

Extensive graft-derived recovery has been observed in relation to several other lesion-induced behavioural deficits, apart from drug-induced rotation. These include tests of hypoactivity, contralateral sensory neglect, and learning deficits (Dunnett et al 1981a, 1983, 1986, 1987; Fray et al 1983), recovery from which depends critically on the exact location of the grafts within the denervated striatum (Dunnett et al 1983). It is conceivable that graft-induced recovery from some of these lesion-induced deficits, such as sensory neglect and spontaneous alternation in a T-maze (Dunnett et al 1983), might reflect spontaneous activity of the foetal grafts more accurately than the drug-induced rotation tests used in this study.
There are some lesion-induced deficits which have not so far been ameliorated by DA cell grafts placed into the striatum. These include skilled independent paw use in reaching tasks (Dunnett et al 1987), and adipsia and aphagia which follow a bilateral lesion (Dunnett et al 1981b, 1983). It should be noted that a small reduction in fluid intake was observed in this study in rats with a unilateral 6-OHDA lesion, which was not corrected by foetal DA cell grafts. The reasons for the ineffectiveness of foetal grafts in ameliorating such lesion-induced deficits are unknown, but could be related to the fact that the grafts are placed into the striatum (rather than into the SN), where they may not receive the full complement of afferent connections that are received by DA cells in the SN. In addition, lesion-induced regulatory deficits appear to be prevented by foetal grafts implanted into the striatum of neonatal rats (Rogers et al 1990), which may suggest that the neonatal brain allows more complete integration of foetal grafts than is possible in adult brain.

It is at present uncertain which of the deficits induced by lesions in rats have the closest correspondence to the functional deficits characterising Parkinson's disease.

7.2. VIABILITY OF GRAFTS IMPLANTED INTO THE 6-OHDA LESIONED STRIATUM

7.2.1. TH immunohistochemistry of foetal grafts
DA cell survival in foetal grafts was examined using tyrosine hydroxylase (TH) immunohistochemistry, which allows cell numbers within the grafts and graft volume to be assessed. However, this method does not give any further indication of the viability of surviving DA cells. It is possible, therefore, that L-DOPA treatment might have damaged implanted DA cells without causing cell death, although there was no indication from cell morphology that this had occurred. One way of obtaining
further information about the viability of cells in the grafts might be to assess levels of TH mRNA using *in situ* hybridisation. Thus, Javoy-Agid et al (1990) found that mRNA levels for TH were reduced in nigral DA cells remaining in patients with Parkinson's disease compared with age-matched controls. Similarly, Pasinetti et al (1989) found that in rats TH mRNA levels were reduced in the DA cells remaining in the SN after a 6-OHDA lesion. Solberg et al (1989) found a discrepancy between the number of DA cells identified in foetal DA grafts by measuring mRNA levels for TH and the number identified by TH immunohistochemistry. A larger number of cells were found using *in situ* hybridisation, suggesting that this is a more sensitive technique. These findings suggest that measurements of mRNA levels might identify DA cells with reduced viability.

7.2.2. Glial reaction within and around foetal grafts

An attempt to obtain an additional index of the viability of the grafts was made by studying the glial reaction within and around the implanted tissue (Chapter 6). As it turned out, interpretation of the glial data was not simple. Thus, whilst all foetal grafts were associated with a gliosis in the surrounding striatum, in both L-DOPA treated and untreated animals, the glial reaction within the grafts was found to be greater in rats treated with L-DOPA. This would have been easy to interpret if the greater glial reaction had been associated with a reduction in survival of TH-positive cells, or with a reduction in graft volume. However, this was not the case and there was no indication that the gliosis was detrimental to graft survival or function.

Knowledge of the role of astrocytes both within foetal cell transplants and in the surrounding host tissue is limited. It is uncertain whether the increased glial reaction found in the implanted host striatum (of both L-DOPA treated and untreated animals) was an encouraging
finding or not. As outlined by Kimelberg and Norenberg (1989), astrocytes have many important roles in the developing and mature CNS, including the metabolism of glutamate and gamma amino-butyric acid (GABA) — both of which are neurotransmitters in the striatum (Albin et al 1989). Astrocytes are also important in the formation of the BBB, as capillaries in the brain are normally surrounded by the 'end feet' of astrocytic fibres (Goldstein and Betz 1986). Such an arrangement was identified in the present study both within the grafts and in the surrounding striatum. Glial cells may also be important in promoting the development and migration of foetal nigral DA cells (Denis-Donini et al 1984; Schults et al 1990). The presence of the astrocytes found in this study could therefore be indicative of an attempt to normalise the milieu within and around the grafts.

The fact that the glial reaction was increased only in the implanted striatum, that it was greatest in the vicinity of the grafts, and that the distribution of glia within the grafts seemed to follow that of TH-positive cells may suggest that the glia serve some function directly related to the metabolism of L-DOPA or DA by the implanted cells. Astrocytes are an important source of monoamine oxidase type B (MAO-B), and their relationship with DA nerve terminals may be important in the degradation of DA released by nerve terminals (Levitt et al 1982). This would be of particular importance if the implanted neurones lacked autoregulatory control. Such a role for glia in this context may explain why they were found in greater abundance in the striatum of animals receiving foetal DA cell grafts than in those receiving sham grafts, and why the grafts of animals treated with L-DOPA contained a higher glial density than those from untreated animals. Grafts in animals treated chronically with L-DOPA may be producing greater amounts of DA than those not exposed to this treatment.
There are, however, more worrying possible explanations for the gliosis around the grafts and within the grafts of animals exposed to L-DOPA. Thus, astrocytes located in the SN may be important in the neurotoxic effects of MPTP, by virtue of their MAO-B content, which oxidises MPTP to its active metabolite MPP⁺ (Levitt et al 1982). This would be consistent with the finding that astroglial ablation can prevent MPTP-induced nigral cell death (Takada et al 1990). If Parkinson's disease is caused by some toxin resembling MPTP, the accumulation of astrocytes in and around grafts may potentially be harmful.

7.2.3. Foetal graft location
The nature of the local environment might influence DA cell survival and longevity. Indeed, there is evidence that local support cells in the SN are crucial for the survival of foetal DA cells (O'Malley et al 1991). If such support cells were reduced in or absent from the striatum, implanted DA cells might be at a disadvantage. A further factor which may affect graft viability is whether DA cells placed 'ectopically' into the striatum have a different susceptibility to toxic effects (whether due to the disease process and/or to the administration of L-DOPA) than DA cells in the SN. MPTP is known to be toxic to DA neurones in the SN, but cells in the VTA are less affected (Waters et al 1986). The reason for this regional variation is unknown. However, it could indicate that different sites in the brain rather than inherent differences in the DA neurones determine the susceptibility of DA neurones to toxins. If this is so, it is possible that DA cells in the striatum might have a different susceptibility to toxins than cells in the SN (such as MPTP, toxic L-DOPA metabolites, or toxic substances that might cause Parkinson's disease). Future studies might usefully assess the effects of probing dopamine cell grafts placed into the striatum with known toxins, such as MPTP.
7.3. SPECIES, NEUROMELANIN AND AGE DIFFERENCES AFFECTING SUSCEPTIBILITY TO TOXIC SUBSTANCES

The results presented in this thesis suggest that rat foetal dopamine cells implanted into the 6-OHDA lesioned striatum can survive to produce functional effects when exposed to L-DOPA and carbidopa, even for prolonged periods. However, transplantation for Parkinson's disease involves the use of human foetal tissue. It is known that there are species differences in the susceptibility of DA neurones to some toxic substances. Thus, unlike human SN DA cells, rat SN DA cells are relatively resistant to damage by MPTP (Boyce et al 1984; Johannessen et al 1985). By analogy, therefore, it is possible that rat DA cells might be more resistant than human DA cells to possible toxic metabolites of L-DOPA. Clearly this question needs to be addressed using human foetal tissue in animal experiments. Failing that, the effect of L-DOPA treatment on the survival of non-human primate foetal dopamine grafts should be studied.

However, the observation that foetal grafts can result in symptomatic improvement in patients receiving L-DOPA (Madrazo et al 1988, 1990a,b; Hitchcock et al 1988, 1990, 1991; Lindvall et al 1990; CR Freed et al 1990) and the indirect evidence of graft survival using \textsuperscript{18}F-DOPA PET scanning (Lindvall et al 1990) suggests that treatment with L-DOPA does not prevent human foetal tissue from surviving.

Another factor which may have some bearing on neuronal susceptibility to toxic substances is the presence of neuromelanin. The neuromelanin content of DA cells varies between species. Adult rat DA cells do not contain neuromelanin, whereas in man neuromelanin increases with age from about 18 months of age (Foley and Baxter 1958; Mann and Yates 1974; Marsden 1983). Cells containing neuromelanin are preferentially lost in the SN of patients with Parkinson's disease (Hirsch et al 1988).
So, if neuromelanin determines the vulnerability of DA cells to the process(es) causing Parkinson's disease, it is conceivable that it might also influence the susceptibility of such cells to toxic metabolites of L-DOPA. The absence of a toxic effect of L-DOPA on the foetal DA cell grafts in this study might be due solely to their lack of neuromelanin. By the same token, lack of neuromelanin in human foetal DA cells may be protective, at least for the first 18 months after implantation.

There is increasing evidence to suggest that the age of CNS tissue affects the susceptibility of neurones to some toxins. Thus, foetal or young adult CNS cells are more resistant to neurotoxic damage than their older counterparts (Jarvis et al 1985; Tulipan et al 1988), whilst recovery rates of TH and DA levels following damage by MPTP are retarded in ageing animals (Nishi et al 1989). Melamed and Rosenthal (1989) found that foetal nigral DA cells in mice were resistant to the toxic effects of MPTP. This was attributed to the absence in the foetal brain of the enzyme MAO-B, which converts MPTP to its active metabolite, MPP+. Recently, however, early postnatal rat dopaminergic systems have also been shown to be resistant to MPP+, suggesting that there is some other difference between immature and adult/ageing tissue which determines their susceptibility to toxins (Feenstra et al 1990).

By analogy, therefore, it is possible that toxic effects of L-DOPA treatment or of the process(es) causing Parkinson's disease were not observed in the rat foetal tissue (and might not be observed in human foetal tissue) implanted into the striatum because of an intrinsic resistance of foetal brain tissue to neurotoxic damage. If this resistance diminishes with age, it is possible that toxic damage might develop in grafts as they mature into older cells. Relevant to this possibility is the fact
that the ageing process of foetal dopamine grafts placed into an 'ectopic' position (i.e. the striatum instead of the substantia nigra) may not be normal, and could even be accelerated. Gopinath et al (1991) observed cytological changes in rat foetal DA cell grafts placed into the striatum which suggested premature ageing. Furthermore, a post mortem study of a patient with 'end-stage parkinsonism' who received a human foetal graft cryopreserved prior to implantation (Redmond et al 1990), may indicate that the implanted cells had undergone an accelerated ageing process. Thus, although only one TH-positive neurone was identified in the implanted striatum, there were cells in the graft which contained neuromelanin, suggesting that they were dopaminergic. The early appearance of neuromelanin in the implanted cells may reflect metabolic overactivity and premature ageing (Mann and Yates 1974).

It is thus possible that toxic effects of L-DOPA treatment are not apparent in the early stages of foetal graft survival (when cells may, by virtue of their immaturity, be inherently more resistant to toxic damage), but could become apparent as the cells age, especially if ageing is accelerated. However, in Chapter 6, the effect of long-term treatment with L-DOPA and carbidopa on implanted foetal DA cells was assessed. Those grafts were studied with TH immunohistochemistry approximately 38 weeks after implantation surgery. Had the implanted DA cells developed in situ in the SN, they would have matured into adult cells by the time of study. There was no evidence from that study, however, that L-DOPA treatment was detrimental either to the functional effects of the ageing grafts or to their survival as revealed by TH immunostaining. Indeed, there is increasing evidence that foetal striatal tissue implants can protect host tissue from subsequent excitotoxin- (Tulipan et al 1989; Pearlman 1991) and 6-OHDA-induced lesions (Przedborski et al 1991), although sham
transplants may have a similar effect on 6-OHDA induced toxicity (Przedborski et al 1991).

7.4. L-DOPA TREATMENT REGIME

7.4.1. Timing of L-DOPA treatment
Another difference between the experimental design used in this study and the situation found in man consists in the L-DOPA treatment regime. Patients receiving foetal grafts will have had chronic DA denervation of the striatum, and are likely to have been receiving L-DOPA (and possibly other drugs as well) for long periods prior to implantation of foetal tissue. Prolonged treatment with L-DOPA prior to implantation of foetal DA cells might indirectly, through an effect on post-synaptic DA receptors, have an adverse effect on graft survival and fibre outgrowth (Lankford et al 1988). As the aim of this study, however, was to assess the direct toxic effects of L-DOPA on implanted cells, treatment was started only following surgery with the result that any such indirect effects of L-DOPA treatment on graft survival, fibre outgrowth or functional activity would be minimised.

7.4.2. Route of administration of L-DOPA
The L-DOPA and carbidopa doses and regime used in this study have been widely used in this laboratory for both short- and long-term treatment of rats (Taylor 1990; Rose 1991). The doses chosen were those that produced similar plasma levels in rats to those found in patients treated with L-DOPA (Hare et al 1973). Oral administration of the drugs in the drinking water was chosen to simulate the clinical situation, and to avoid repeated injections of these drugs. The most commonly used clinical regime, however, involves oral doses divided over an 18-24 hour period. This regime would have been more accurately reproduced in the laboratory by administering oral boluses of the drugs, several times a day, but this was
impracticable when the number of animals and duration of treatment were taken into account. Oral administration of the drugs in the rats' drinking water should mean that highest plasma levels of the drug were achieved during the dark cycle, when maximum drinking occurs. Lower levels would have been obtained during the day. It is to be expected that with this regime the plasma profile of L-DOPA would show smaller peaks and troughs than would be achieved by intermittent injections of the drugs. Conversely, the plasma profile of L-DOPA using this regime would not be as smooth as that obtained by continuous infusion of the drugs.

The type of L-DOPA treatment regime employed is important as it may determine the temporal pattern with which L-DOPA (and ultimately DA) reaches the denervated striatum. This pattern affects DA-agonist mediated behaviours (Juncos et al 1989), DA receptor densities (Ariano et al 1991) and levels of neurotransmitters in the basal ganglia (Engber et al 1991).

In one study, rats with a unilateral 6-OHDA lesion receiving intermittent L-DOPA treatment showed increased rotational responses to the mixed D-1/D-2 agonist apomorphine, whereas rats receiving continuous L-DOPA treatment did not (Juncos et al 1989); further increases in D-1 binding density were observed in the lesioned striatum of rats receiving continuous L-DOPA treatment, but no significant change was seen in those treated intermittently. Weick et al (1990) studied the behavioural response of 6-OHDA lesioned rats to the D-1 agonist SKF 38393 following treatment with L-DOPA, and found that intermittent L-DOPA treatment reduced the rotational response to D-1 agonist but continuous treatment did not; these behavioural changes occurred without any corresponding changes in D-1 or D-2 receptor densities.
Recently, Ariano et al (1990, 1991), using combined immunohistochemical and autoradiographic studies reported that a 6-OHDA lesion resulted in an ipsilateral decrease in D-1 density, together with a dissociation between binding sites and the post-synaptic cAMP-positive neurones. They reported that continuous treatment (ip infusion) with L-DOPA resulted in re-association of D-1 binding sites with striatal cells in the dorsomedial striatum, together with an increase in number of D-1 receptors in the lesioned striatum. However, Ariano et al (1991) noted that, whilst intermittent treatment (twice daily ip injections) with L-DOPA resulted in a similar re-association of D-1 sites and cAMP-positive neurones in the dorsomedial striatum, this treatment was associated with a decrease in overall D-1 density in both the intact and lesioned striata. There is also evidence that different L-DOPA treatment regimes have different effects on neurotransmitter levels in the basal ganglia. Engber et al (1991) reported that 6-OHDA lesion-induced increases in striatal glutamic acid decarboxylase (GAD) activity and globus pallidus enkephalin content were differently affected by different L-DOPA regimes. Thus, intermittent L-DOPA treatment (twice daily injection) further increased GAD activity and had no effect on enkephalin content, whilst continuous infusion of L-DOPA further elevated enkephalin content, without affecting other neurochemical markers.

The way in which L-DOPA is delivered to the striatum, therefore, clearly affects many aspects of basal ganglia function. Conceivably, different L-DOPA treatment regimes may also influence the functional effects of foetal grafts, neurotransmitter expression, cell survival and/or fibre outgrowth. Hence the conclusions drawn from this study must be confined to the particular drug treatment regime used here.
7.4.3. Do toxic metabolites of L-DOPA reach the implanted cells?
With any L-DOPA treatment regime, unless in vivo dialysis is performed, there is uncertainty about how much of the drug actually reaches the denervated striatum, and how much is converted into DA. The conclusion that L-DOPA treatment was not harmful to foetal VM cells implanted into the 6-OHDA lesioned striatum is based on the assumption that some DA (and consequently its potentially toxic metabolites) actually reached those implanted cells. The fact that animals treated with L-DOPA demonstrated contralateral rotation in their cages during the first 2 weeks of treatment suggests that some DA was indeed reaching the denervated striatum. Similar behaviour has been observed by others in L-DOPA-treated 6-OHDA lesioned rats (Karoum et al 1988). Furthermore, there is considerable evidence from other studies that exogenously administered L-DOPA is converted into DA in the 6-OHDA lesioned striatum. L-DOPA crosses the BBB and is converted into DA by dopa decarboxylase, predominantly in dopaminergic elements. However, dopa decarboxylase also exists in non-aminergic striatal cells, so that even after massive lesions of the nigrostriatal pathway significant amounts of the enzyme remain in the striatum with the effect that conversion of L-DOPA to DA may still occur (Melamed and Hefti 1984).

There is direct evidence, using intracerebral dialysis, that L-DOPA is converted into DA in the 6-OHDA lesioned striatum (Zetterstrom et al 1986). These authors reported that administration of L-DOPA (20mg/kg ip, without a peripheral dopa decarboxylase inhibitor) to rats produced peak increases in extracellular DA in the 6-OHDA lesioned striata equal to the increases found in the intact striata. Abercrombie et al (1990) reported that administration of L-DOPA (100mg/kg ip, with a peripheral dopa decarboxylase inhibitor) to rats with virtually complete 6-OHDA lesions resulted in a greater
increase in extracellular DA than that observed in intact animals. These authors suggested that intact DA nerve terminals may provide a 'sink' for administered L-DOPA, and that the therapeutic efficacy of L-DOPA in Parkinson's disease results from its conversion in to DA in non-dopaminergic striatal elements. Thus, there is indirect (behavioural) and direct (dialysis) evidence that exogenously administered L-DOPA results in significant (or even 'supranormal') amounts of DA in the 6-OHDA lesioned striatum. It is reasonable to assume, therefore, that foetal VM grafts would be exposed to any toxic by-products of L-DOPA metabolism.

7.5. FUTURE DIRECTIONS

The foregoing discussion suggests a number of possible directions for studies of the effects of L-DOPA treatment on the survival and function of foetal VM grafts. As indicated above, questions which might be investigated are the effect of L-DOPA treatment on the survival of foetal DA cells implanted into the partially-lesioned striatum, the effect of chronic treatment with L-DOPA for long periods prior to the implantation of foetal cells, and the effect of a long-standing lesion.

In addition, the question of graft location may be important in determining sensitivity to toxic substances (such as may result from a disease process or from the metabolism of L-DOPA). Future studies could assess the effects of exposing foetal grafts implanted into a variety of different locations to known neurotoxins, such as MPTP (in the primate) and MPP⁺ or 6-OHDA (in the rodent). Graft location may also be crucial to the recovery of certain lesion-induced deficits of relevance to man. In theory, a graft placed within the SN would be expected to have the greatest chance of re-forming normal reciprocal connections with host systems, and thus of causing recovery from the broadest range of deficits.
Attempts to obtain functional recovery and reinnervation of the striatum by DA cell grafts placed into the 6-OHDA-lesioned SN have been unsuccessful, however, although 'bridges' made of peripheral nerve or striatal tissue have enhanced dopamine fibre outgrowth from grafts placed in the SN (Aguayo et al 1984; Dunnett et al 1989). Further work in this area should be undertaken.

Graft connectivity with host neuronal circuitry is another area where further studies are required. In this study, the question of DA cell connectivity with host neurones was not investigated. Ultrastructural and tracer transport studies (using tracer substances such as horseradish peroxidase and phaseolus vulgaris) have shown that foetal VM grafts can form efferent synaptic connections with host neurones, and receive some afferent inputs. Whether these connections are physiologically appropriate or normal is uncertain. Freund et al (1985) and Clarke et al (1988) demonstrated synaptic connections between transplanted TH-positive neurones and the adjacent host neurones at the host-graft interface. Freund et al (1985) reported that a proportion of the synapses appeared abnormal (in particular those made with magnocellular interneurones of the striatum), and they failed to find any evidence of afferent input from the host into the grafted tissue. However, Doucet et al (1989a) demonstrated host afferents from the ipsilateral cortex and striatum, but only into the peripheral regions of the grafts. Nishino et al (1990) demonstrated mutual synaptic connections between TH-positive elements in foetal VM grafts and TH-negative elements in the host striatum, which persisted for up to 2 years. According to Doucet et al (1989a), the integration of foetal grafts with the host neuronal circuitry varies with the age of the recipient, the site of implantation, the type of graft, and the extent of damage at the implantation site. Conceivably, therefore, L-DOPA treatment may also affect the integration of implanted DA cells with host neuronal
circuitry. The effect of such treatment at the synaptic level should be evaluated.

A further area of considerable importance is that of growth factors. At present the factors controlling growth of foetal DA cells implanted into the striatum are unknown. To date, there have been no reports of excessive neuronal proliferation, although numerous reactive astrocytes have been observed in and around foetal DA cell grafts (Gopinath et al 1991; and the present results, Chapter 6). Until recently, trophic factors promoting the survival of mesencephalic DA neurones defied detection. The recent demonstration that Brain-Derived Neurotrophic Factor (BDNF) is trophic for mesencephalic DA neurones, and that it protects cultured nigral cells from the neurotoxic effects of MPP⁺ (Hyman et al 1991), opens an avenue for investigating the effects of BDNF on DA cell grafts. For example, cografts of cells genetically modified to produce BDNF together with foetal DA cells might provide a long-lasting source of DA, together with 'in-built' protection from some neurotoxins.

Further knowledge of the mechanism(s) of graft action is needed. Thus, although animal experiments indicate that specific graft-host synapses may be important in determining behavioural recovery, a diffuse release mechanism could also be important (Bjorklund et al 1988). It is therefore interesting that a number of the patients who have received foetal nigral grafts into one side of the brain have shown bilateral symptomatic improvement (Madrazo et al 1988, 1990; Lindvall et al 1990; Hitchcock et al 1988, 1990, 1991; CR Freed et al 1990), suggesting that diffuse release of DA may be occurring. In addition, recent reports of symptomatic improvement and host DA fibre regeneration in patients receiving adrenal medulla grafts (Kordower et al 1991; Olson et al 1991) suggest that certain kinds of transplants may have a
therapeutic effect via an unknown mechanism.

As well as the question of the effects of drug treatment on graft survival, the question of whether immunosuppressive treatment is needed for patients receiving foetal grafts needs to be resolved. Other important areas for further work include the best means of collection and storage of foetal DA tissue for use in transplants at a later date. In order to simulate more closely the human situation, further research should be carried out with primate models of Parkinson's disease, preferably using human foetal tissue grafts.


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285


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