Manipulation of precursor cells for the replacement of complex circuitry lost in neurodegenerative disease

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Summary

The research reported in this thesis focused on the potential of neural progenitor cells to provide a suitable source of neurones which can be used in cell replacement strategies for Parkinson's disease. Specifically, the parameters affecting the differentiation of these cells into neuronal phenotypes were addressed and increasing the survival of transplanted dopamine neurones was attempted. In addition, the *in vitro* capacity of adult neural progenitor cells to generate neurones was assessed and a mouse model of Parkinson's disease was established.

In Chapter Three an extensive study investigating the effects of donor age and periods of *in vitro* proliferation on the neurogenic capacity of foetal rat neural progenitor cells revealed that these two parameters had significant effects on neuronal differentiation. While ventral mesencephalic (VM) cells isolated at embryonic day 12 generated the most neurones, increased periods of *in vitro* cell expansion had detrimental effects on neuronal yield. Dopamine differentiation was also severely effected by *in vitro* proliferation with VM cells failing to generate any dopamine neurones even after short-term expansion.

In Chapter Four, in an attempt to increase the survival rate of transplanted dopamine neurones, dopamine neurones were transplanted into the striatum of rat models of Parkinson's disease in a solution containing the antioxidant, ascorbic acid. Dopamine neurones transplanted with ascorbic acid formed grafts containing more dopamine neurones compared to standard dopamine grafts, indicating a specific survival effect of ascorbic acid.

The ability of adult neural progenitor cells to generate neurones has generated great excitement over the past years, however, while these cells have shown the capacity to generate neurones, the ability of these cells to differentiate into neurones of dopaminergic phenotypes has not been demonstrated. This issue was addressed in Chapter Five, where both expanded and non-expanded adult progenitors were assessed for the expression of the non-specific dopamine marker tyrosine hydroxylase (TH). Neither expanded nor non-expanded cells that differentiated into neurones were immunopositive for TH, thus indicating an inability of these cells to spontaneously differentiate into dopamine neurones.

To efficiently assess the functional capacity of mouse-derived embryonic stem cells, a mouse model of Parkinson's disease was established in Chapter Six. Rotational behaviour induced following amphetamine and apomorphine challenge in mice with either unilateral 6-OHDA lesions of the medial forebrain bundle (MFB) or the striatum were compared. While both models showed rotational bias, MFB lesions were variable and unreliable, whereas more consistent dopamine loss was observed following striatal lesions. The striatal lesion therefore provided the better lesion in mice, and optimal rotational bias was elicited following 10mg/kg amphetamine challenge.

To confirm rotational behaviour in this mouse model reflected dopamine loss, in Chapter Seven, mice received intrastratal transplants of dopamine neurones. 4-6 weeks post-transplantation, significant attenuation of amphetamine-induced rotational bias was observed. This behavioural recovery not only confirmed that rotations reflect dopamine loss, but also demonstrated the suitability of this model for measuring the functional capacity of mouse-derived cell lines.
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>6-OHDA</td>
<td>6-Hydroxydopamine</td>
</tr>
<tr>
<td>CAPIT</td>
<td>Core Assessment Protocol for Intracerebral Transplantation</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Cpu</td>
<td>Caudate-Putamen</td>
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<tr>
<td>CRL</td>
<td>Crown Rump Length</td>
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<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>CTX</td>
<td>Cortex</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GPe</td>
<td>Globus Pallidus (external segment)</td>
</tr>
<tr>
<td>GPi</td>
<td>Globus Pallidus (internal segment)</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks buffered saline solution</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>IL-</td>
<td>Interleukin</td>
</tr>
<tr>
<td>L-dopa</td>
<td>Levodopa</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukaemia inhibitory factor</td>
</tr>
<tr>
<td>MFB</td>
<td>Medial forebrain bundle</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MTN</td>
<td>Medial terminal nucleus of the accessory optic tract</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>NK</td>
<td>Neuman Keuls statistical test</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia nigra</td>
</tr>
<tr>
<td>SNC</td>
<td>Substantia nigra pars compacta</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>SNr</td>
<td>Substantia nigra <em>pars reticulata</em></td>
</tr>
<tr>
<td>STN</td>
<td>Subthalamic nucleus</td>
</tr>
<tr>
<td>TBS</td>
<td>TRIS buffered saline</td>
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<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TNS</td>
<td>TRIS non-saline</td>
</tr>
<tr>
<td>TRIS</td>
<td>Trizma base</td>
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<tr>
<td>VM</td>
<td>Ventral mesencephalon</td>
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<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>WGE</td>
<td>Whole ganglionic eminence</td>
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Chapter One

Introduction

This thesis discusses the potential use of neural progenitor cells as an alternative to foetal tissue in neuronal replacement therapies for Parkinson's disease. The research concentrates on generating sufficient numbers of dopamine neurones from progenitor cells by investigating the effect of donor age and prolonged proliferation on cells isolated from the developing rat ventral mesencephalon (VM). In addition, increasing the survival of dopamine neurones by the addition of the antioxidant ascorbic acid (Vitamin C) to the culture environment of primary VM neurones has been assessed, and a discussion on the use of adult neural stem cells as a potential donor source of cells for transplantation is also included. A 6-OHDA-lesion model of Parkinson's disease has been established in mice, which displays appropriate rotational behaviour reflecting unilateral dopamine depletion, and the attenuation of rotational bias following reinnervation of the striatum by intrastriatal transplantation of dopamine neurones. This model allows the assessment of the in vivo potential of mouse-derived embryonic stem (ES) cells, and other mouse cell lines without the complications associated with immunosuppression.

The following chapter describes the basic anatomy and function of the basal ganglia, the degeneration of this structure with respect to Parkinson's disease, current pharmacological and surgical treatments for this neurodegenerative disorder, and a detailed discussion on stem cells and their potential in providing a source of neurones for neurotransplantation.

1.1 The Basal Ganglia

The basal ganglia are a structure consisting of a number of subcortical nuclei that have numerous interconnections and pathways. The connectional anatomy of the nuclei is highly complex, with pathways having excitatory or inhibitory functions and utilising a variety of neurotransmitters. The following description of the basal ganglia
Chapter One outlines the main nuclei and the major pathways that collectively play an important role in the regulation of motor and cognitive control.

1.1.1 Anatomy of the basal ganglia

The striatum, globus pallidus, subthalamic nucleus and the substantia nigra are the main nuclei that form the basal ganglia (Fig. 1.1). The striatum is comprised of two regions, namely the dorsal zone (neostriatum) and ventral zone (ventral striatum). The neostriatum is composed of the putamen and caudate nuclei while the ventral striatum is composed of the nucleus accumbens and the olfactory tubercle. The globus pallidus, also known as the pallidum, consists of external and internal segments (GPe & GPi respectively), with the GPe positioned adjacent to the putamen, and the GPi situated medial to the GPe. The combination of the putamen and the globus pallidus is referred to as the lenticular or lentiform nucleus. Like the pallidum, the substantia nigra is also divided into two parts, namely the pars reticulata (SNr) and pars compacta (SNC) (Fig. 1.1). The basal ganglia link the cortex to the thalamus and its main inputs are from excitatory glutamatergic cortical neurones that project to the striatum. Striatal efferents contain different neurotransmitters and project to various nuclei, but ultimately terminate at the basal ganglia output nuclei (the SNr and GPi). The output nuclei then send inhibitory GABAergic projections to the thalamus, which in turn projects back to the cortex (Fig. 1.2).

| A | Basal Ganglia | = | Striatum | + | Globus Pallidus | + | Substantia Nigra | + | Subthalamic Nucleus |
| B | Striatum | = | Neostriatum (Caudate/Putamen) | + | Ventral Striatum (Nucleus accumbens/Olfactory tubercle) |
| C | Globus Pallidus | = | Globus Pallidus Internal segment (GPI) | + | Globus Pallidus External segment (GPe) |
| D | Substantia Nigra | = | Substantia Nigra Pars Reticulata (SNR) | + | Substantia Nigra Pars Compacta (SNC) |

*Figure 1.1 Diagram showing the main nuclei of the basal ganglia (A). The subdivisions of the Striatum (B), Globus Pallidus (C) and the Substantia Nigra (D) are also shown.*
The basal ganglia connections are organised into a series of functional circuits, with each circuit segregating and relaying specific cortical information to distinct target zones in the thalamus and in turn to defined areas of the cortex (Alexander & Crutcher, 1990). The motor circuit receives input from the sensorimotor cortex and projects to the putamen and then to the output nuclei. The output nuclei then send their efferents to the ventroanterior and ventrolateral nuclei of the thalamus before projecting back to the premotor and supplementary motor cortex. The cognitive circuit receives its input from the association cortex and passes through the caudate nucleus, basal ganglia output nuclei, ventroanterior and ventrolateral nuclei of the thalamus and then projects back to the prefrontal cortex (Behan & Barker, 2001).

As well as these circuits, the circuitry of the basal ganglia is made even more complicated by an internal loop that projects from the striatum to the substantia nigra pars compacta and then back to the striatum, and by two different pathways that project from the striatum to the SNr and GPi, which function to modulate the inhibitory outflow of the output nuclei. One pathway is the inhibitory 'direct' pathway where striatal neurones containing GABA and substance P project directly to the SNr and GPi. The other pathway is the 'indirect' pathway where striatal neurones containing GABA and enkephalin project to the GPe, then to the subthalamic nucleus via a GABAergic pathway, and finally to the SNr and GPi via excitatory glutamatergic neurones (Fig. 1.2). Activation of the direct pathway causes disinhibition of the thalamus and results in excessive excitation of the cortex. Alternatively, activation of the indirect pathway causes disinhibition of the subthalamic nucleus, which results in excitation of the output nuclei, inhibition of the thalamus and overall inhibition of the cortex (Alexander & Crutcher, 1990; Albin et al., 1989). The two pathways therefore have opposing effects and a fine balance exists between them. This balance is regulated by the dopaminergic nigrostriatal projection neurones, which function by having an inhibitory effect on the striatopallidal pathway and a excitatory influence over the striatonigral neurones (Gerfen, 1992). Evidence for this opposing dopaminergic regulation has come from studies assessing dopamine-related glucose utilisation of the globus pallidus and substantia nigra via 2-deoxyglucose autoradiography (Engber et al., 1990), as well as from studies examining the electrophysiological activity of the globus pallidus (Bergstrom et al., 1982) and substantia nigra (Waszczak et al., 1984). In addition, dopamine has been shown to regulate the expression of enkephalin in the striatopallidal neurones and...
substance P & dynorphin in the striatonigral neurones, as demonstrated following neurotoxin-induced dopamine denervation of the striatum. In such studies, increased enkephalin mRNA expression in the striatopallidal neurones and decreased expression of substance P and dynorphin mRNA in striatonigral neurones are observed following neurotoxin-induced destruction of the nigrostriatal pathway (Gerfen et al., 1990; 1991; Gerfen, 1992; Normand et al., 1988; Graybiel, 1990; Voorn et al., 1987; Young III et al., 1986).

Figure 1.2 Schematic diagram of the circuitry and the neurotransmitters of the basal ganglia-thalamocortical circuitry, showing the ‘indirect’ and ‘direct pathways’ from the striatum to the output nuclei. Inhibitory neurones are shown in black and excitatory neurones in grey. Abbreviations: DA, dopamine; Enk, enkephalin; GABA, \( \gamma \)-aminobutyric acid; GPe, external segment of the globus pallidus; GPi, internal segment of the globus pallidus; Glu, glutamate; PPN, pedunculopontine nucleus; SNC, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; SP, substance P; STN, subthalamic nucleus. (Adapted from Alexander & Crutcher, 1990).
1.1.2 Patch/matrix organisation of the striatum

The striatum is a highly organised structure that processes information at a number of different levels (Gerfen., 1992). One of the levels of organisation is the patch/matrix compartmentalisation of the striatum, which was revealed following histochemical staining for the enzyme acetylcholinesterase (AChE) (Graybiel & Ragsdale., 1978). The patch compartments or striosomes, which were first defined as areas corresponding to AChE-poor regions in cats and monkeys, are embedded in the AChE-rich matrix. The patch compartments also correspond to opiate receptor binding-sites and exhibit high levels of immunoreactivity for enkephalin and substance P (Graybiel., 1990), whereas the matrix compartment can be distinguished by somatostatin immunoreactivity and neurones expressing the calcium-binding protein, Calbindin (Gerfen et al., 1985; 1987b).

The patch and matrix compartments both have distinct input-output connections. The striatal afferents are organised into patch/matrix compartments and come from 3 main areas; the thalamic intralaminar complex, the cortex and the midbrain. The patch compartment has been shown to receive innervation from thalamic fibres arising from the centromedian nucleus (Kalil., 1978; Royce., 1978), cortical efferents from the deep layer V and layer VI of the cortex (Gerfen., 1989; 1992) and dopamine projections from the ventral substantia nigra pars compacta and substantia nigra pars reticulata (Gerfen et al., 1987a; 1987b; Gerfen., 1989). The matrix compartment however, receives innervation from thalamic parafasicular nucleus (Gerfen., 1984; 1985), cortical innervation from superficial layer V and supragranular layers of the cortex (Gerfen., 1989; 1992) and midbrain dopaminergic efferents from the ventral tegmental area (VTA, A10 cell group), the retrorubral area (A8) and the dorsal substantia nigra pars compacta (A9) (Gerfen., 1989). The outputs of the patch and matrix compartments are via medium spiny neurones, which predominately innervate the ventral dopamine neurones of the substantia nigra pars compacta and the entire substantia nigra pars reticulata respectively (Gerfen., 1984; 1985).

1.2 Diseases of the basal ganglia

Normal function of the basal ganglia is dependent on the balance between the direct and indirect pathways that regulate the inhibitory outflow of the output nuclei. Imbalance between these two pathways has been suggested to lead to a variety of motor disorders including Parkinson’s and Huntington’s disease (Albin et al., 1989;
Delong., 1990). In Parkinson’s disease, the degeneration of the nigrostriatal dopamine neurones of the pars compacta results in increased inhibitory outflow of striatal neurones to the GPe, which causes disinhibition of the subthalamic nucleus and therefore increased activity of the output nuclei. This increased activity ultimately results in inhibition of the thalamus and the cortex resulting in the hypokinesia (reduced levels of movement) of Parkinson’s disease (Jenkins et al., 1992; Hutchison et al., 1998) (see Fig. 1.2). In Huntington’s disease there is degeneration of the striatal neurones that project to the GPe, causing reduced activity of the output nuclei. This in turn, causes disinhibition of the thalamus and excessive excitation of the cortex resulting in hyperkinesia (excessive movement) (Albin et al., 1989)

1.2.1 Parkinson’s disease

Initially described by James Parkinson in 1817 in his Essay on the shaking palsy, Parkinson’s disease (PD) is a progressive, neurodegenerative disorder, which is second in prevalence only to Alzheimer’s disease. The incidence of PD increases with age, with approximately 2% of the population over the age of 65 being affected, rising to over 4% at the age of 85 and above (see reviews by Arenas., 2002, Kolchinsky., 2001). PD is however, not essentially a disease of the aged, with onset in 3-10% of PD patients occurring before the age of 40 (Tsai et al., 2002).

Parkinson’s disease is primarily characterised by the loss of dopaminergic nigrostriatal projection neurones (particularly the ventrolateral portion of the A9 cell group), leading to a large reduction in dopaminergic innervation of the striatum, as can be visualised by the loss of [18F]dopa uptake in the striatum by using positron emission tomography (PET) (Sawle et al., 1993). Specifically, the depletion of dopamine results in decreased innervation of the putamen, which is principally the “motor” division of the striatum, whereas the dopaminergic innervation of the caudate nucleus, which is more associated with cognitive function, is less affected (Agid., 1991). In addition to the loss of dopamine, another pathological characteristic of PD is the presence of intracellular inclusions termed Lewy bodies in the remaining neurones (although these are not specific to Parkinson’s disease) (Spillantini et al., 1997; Duda et al., 2000; Higuchi et al., 2004).

The disease symptoms only appear when 80% of striatal dopamine or 50% of the nigral cells are lost (Au et al., 2005) and predominantly result in a catalogue of movement impairments including bradykinesia (slowness of movement), rigidity (due
to increased muscle tone) tremor (characteristically involving the hands in a "pill-rolling" movement) and postural abnormalities (Calne et al., 1992; Samii et al., 2004). Symptoms are however, not only associated with motor function and a number of non-motor symptoms are also seen. These include autonomic dysfunction, sensory symptoms, sleep disturbances and cognitive and psychiatric changes (Samii et al., 2004). Cognitive dysfunction in PD is common, in particular dementia, which affects about 40% of patients (Emre., 2003), and depression, which affects nearly half of patients suffering from the disease (Cummings., 1992; Yamamoto., 2001; Allain et al., 2005). Along with the primary loss of dopamine neurones, depletion of noradrenergic neurones of the locus coeruleus (German et al., 1992; Chan-Palay & Asan., 1989), cholinergic neurones of the nucleus basalis of Meynert (Candy et al., 1983) and serotonergic neurones of the raphe (Halliday et al., 1990) are also evident in PD. The loss of these non-dopaminergic neuronal cells have been suggested to contribute to the non-motor symptoms of the disease, with noradrenergic loss being involved in the onset of both depression (Chan-Palay & Asan., 1989) and dementia (Zweig et al., 1993). PD depression is also closely associated to serotonin depletion, with a correlation between depression and reduced cerebrospinal fluid levels of the serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA) (Mayeux et al., 1988).

The aetiology of PD remains unknown, although, various environmental factors have been suggested in the development of the disease (Fuente-Fernández & Calne., 2002; Tsai et al., 2002), with rural living, well water drinking, farming, pesticide exposure (Priyadarshi et al., 2001) and viral infection (Pradhan et al., 1999) implicated as possible risk factors. In addition, genetic predisposition may also play a role in the development of PD (Pankratz & Foroud., 2004; Huang et al., 2004; Gasser., 2001; Cordato & Chan., 2004; Le & Appel., 2004), with a variety of genetic mutations having been discovered (summarised in table 1.1). The first 'PD-gene' to be identified was a gene for the protein α-synuclein, which is normally expressed throughout the brain (Golbe et al., 1990). Interestingly, the presence of abnormal aggregates of this protein is the main constituent of Lewy bodies.

There are currently many treatments available for the management of Parkinson's disease†, with the majority concentrating on providing symptomatic relief through

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† A comprehensive review on the treatments available for the management of Parkinson's disease can be found in Movement Disorders Volume 17 supplement 4, 2000.
drug therapy. Although a number of drugs with different modes of action are available (see review by Hely et al., 2000), pharmacological treatments that increase CNS dopamine levels are the most effective in treating disease symptoms. Some dopamine-enhancing drugs, like Tolcapone (Tasmar®) and Entacapone (Comtan®), aim to inhibit dopamine breakdown and therefore increase the availability of endogenous dopamine. Other drugs act as dopamine agonists, thus directly stimulate dopamine receptors e.g. Bromocriptine (Parlodel®) and Pergolide (Permax®). The most effective and widely used drug in the treatment of PD is however, the dopamine precursor Levo-3,4-dihydroxyphenylalanine (L-dopa). Once administered, L-dopa crosses the blood-brain barrier and is converted to dopamine by dopa-decarboxylase thus increasing brain dopamine levels. However, while L-dopa and dopamine agonists are effective at alleviating symptoms of PD in early stages of the disease, the efficacy of dopamine drug therapy becomes limited by both disease progression and by the development of drug-associated dyskinesia (involuntary movements) (Obeso et al., 2000; Nutt., 2001).

As a result of these limitations, alternative strategies for the treatment of PD are necessary and these alternatives may include a number of surgical procedures. Ablative techniques such as thalamotomy, pallidotomy and subthalamotomy (Obeso et al., 1997) as well as deep brain stimulation of these nuclei (Walter & Vitek., 2004) have been used with varying degrees of success. However, these strategies do not repair nor cure the underlying disease process, in contrast to neural transplantation strategies (see section 1.4) that aim to reconnect the degenerated circuitry caused by the disease.

Table 1.1 Genetics of Parkinson’s disease

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene (if known)</th>
<th>Chromosomal Region</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK 1</td>
<td>α-synuclein</td>
<td>4q21-23</td>
<td>Polymeropoulos et al., 1997; Zarranz et al., 2004</td>
</tr>
<tr>
<td>PARK 2</td>
<td>Parkin</td>
<td>6q25.2-27</td>
<td>Kitada et al., 1998</td>
</tr>
<tr>
<td>PARK 3</td>
<td>?</td>
<td>2p13</td>
<td>Gasser et al., 1998</td>
</tr>
<tr>
<td>PARK 4</td>
<td>?</td>
<td>4p14-16.3</td>
<td>Waters &amp; Miller., 1994</td>
</tr>
<tr>
<td>PARK 5</td>
<td>UCH-L1</td>
<td>4p14</td>
<td>Leroy et al., 1998</td>
</tr>
<tr>
<td>PARK 6</td>
<td>?</td>
<td>1p35-36</td>
<td>Valente et al., 2001; 2002</td>
</tr>
<tr>
<td>PARK 7</td>
<td>DJ-1</td>
<td>1p36</td>
<td>van Duijn et al., 2001; Bonifati et al., 2003a; 2003b</td>
</tr>
<tr>
<td>PARK 8</td>
<td>?</td>
<td>12p11.2-13.1</td>
<td>Funayama et al., 2002</td>
</tr>
<tr>
<td>PARK 9</td>
<td>?</td>
<td>1p36</td>
<td>Hampshire et al., 2005</td>
</tr>
<tr>
<td>PARK 10</td>
<td>?</td>
<td>1p32</td>
<td>Hicks et al., 2002</td>
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<tr>
<td>NURR 1</td>
<td>NR4A2</td>
<td>2q22-23</td>
<td>Le et al., 2002</td>
</tr>
</tbody>
</table>
1.3 Animal models of Parkinson's disease

Replicating Parkinson's disease in animal models can be achieved either by using neurotoxic compounds which destroy nigrostriatal dopamine neurones, or by using genetic engineering to remove/alter or add/overexpress certain genes that are known to be involved in the pathogenesis of PD.

In this thesis, all animal models of Parkinson's disease were established by the destruction of the nigrostriatal dopamine pathway using the neurotoxin 6-hydroxydopamine (6-OHDA). The lesions were unilateral, and depending on the site of neurotoxin injection, lesions of the nigrostriatal pathway were either near total (medial forebrain bundle lesions, MFB) or partial (striatal lesions), resulting in depletion of dopamine in the target structure, the striatum. Since unilateral dopamine depletion of the striatum results in pronounced drug-induced rotational behaviour, the rotation model was used to quantify dopamine loss and to assess functional recovery following transplantation.

1.3.1 Neurotoxin models of Parkinson's disease

Although a number of neurotoxins such as Rotenone and Reserpine have been used to deplete the nigrostriatal dopamine pathway (see reviews by Betarbet et al., 2000; 2002), the two main toxins that are used in animal models of Parkinson's disease are the neurotoxins 6-OHDA or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

1.3.1.1 6-Hydroxydopamine (6-OHDA)

The neurotoxic effect of 6-OHDA was described by the Swedish scientist Urban Ungerstedt who showed the depletion of dopamine cell bodies in the substantia nigra and dopamine terminals in the striatum following stereotaxic injections of this compound into the substantia nigra of rats (Ungerstedt, 1968). The mechanism of 6-OHDA-induced neuronal death is associated with the generation of free radicals and subsequent oxidative stress. 6-OHDA is taken up by dopamine terminals and oxidised to produce hydrogen peroxide, thus damaging the neurones from within (Heikkila & Cohen, 1971; 1973; Cohen & Heikkila, 1974). Although hydrogen peroxide itself is not very toxic, in the presence of iron it can form the harmful hydroxyl radical via the Fenton reaction (Sachs & Jonsson, 1975; Olanow, 1993; Dunnett & Björklund, 1999). In addition, 6-OHDA has also shown to reduce endogenous antioxidants
(Perumal et al., 1992) and increase the production of superoxide free radicals by inhibiting mitochondrial complex I (Cleeter et al., 1992). Stereotaxic injection of 6-OHDA into the area of interest is necessary to induce dopaminergic cell death since 6-OHDA is unable to cross the blood-brain barrier and therefore cannot be given systematically in adult animals.

1.3.1.2 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)
The selective targeting and destruction of midbrain dopamine neurones following MPTP administration was discovered inadvertently following its Parkinsonism-inducing effects in humans (Langston et al., 1983; Ballard et al., 1985). This toxin has since provided an alternative to the use of 6-OHDA, and unlike 6-OHDA can be administered systematically. The toxicity of this compound is mediated by its conversion from MPTP into the toxic by-product 1-methyl-4-phenylpyridinium (MPP+) by monoamine oxidase B. MPP+ is then taken up by the dopamine transporters and accumulates in dopamine neurones where it inhibits mitochondrial complex I, resulting in the generation of reactive oxygen species (Shimohama et al., 2003). Oxidative stress then ensues as evidenced by increased lipid peroxidation (Rios & Tapia., 1987) and decreased antioxidant levels (Sriram et al., 1997). The effects of MPTP however vary across and within species, with rats being resistant to this neurotoxin (Giovanni et al., 1994; Betarbet et al., 2002, Shimohama et al., 2003). MPTP is therefore not used in our present studies.

1.3.1.3 Behavioural effects of neurotoxin-induced degeneration of the nigro-striatal dopamine pathway
As a result of unilateral 6-OHDA lesion-induced dopamine depletion of the nigrostriatal pathway, marked motor disturbances in animals are observed, thus highlighting the importance of dopamine on motor function (Ungerstedt., 1968). This abnormal motor behaviour is characterised by spontaneous circling behaviour or rotations, which are ipsilateral to the lesion (Ungerstedt., 1968; Ungerstedt & Arbuthnott., 1970). Rotational behaviour has been shown to increase in response to stimulatory drugs such as the dopamine releasing drug amphetamine (Ungerstedt., 1971a) and to the dopamine receptor agonist apomorphine (Ungerstedt., 1971b). However, when using apomorphine the direction of rotation is contralateral to the
lesion, indicating supersensitivity of dopamine receptors in the denervated striatum (Ungerstedt, 1971b). Since the degree of rotation correlates well to dopamine loss (Lee et al., 1996), measurement of rotational behaviour gives a good indication of the extent of dopaminergic innervation of the striatum. In addition to the rotation model, unilateral 6-OHDA lesioned rats show contralateral limb akinesia and are unable to initiate stepping movements with the contralateral paw. This initiation deficit can be assessed using the 'stepping test' (Schallert et al., 1992), which shows the ipsilateral paw of 6-OHDA lesioned rats frequently making adjusting steps as it is moved slowly sideways across a table. This is in contrast to the contralateral paw, which passively drags as the rat is moved (Schallert et al., 1992; Olsson et al., 1995). The contralateral paw is also impaired in reaching and grasping tasks when compared to the ipsilateral paw (Olsson et al., 1995), as measured using a simple food retrieval task (Whishaw et al., 1986) or the 'staircase test'. This test is designed to assess skilled forelimb use by placing food on a staircase that is situated on either side of a central platform on which the lesioned animal is placed. The staircase has several graded stages of reaching difficulty, and therefore forelimb extension as well as grasping ability can be assessed (Montoya et al., 1991). Alternatively, limb impairment can be assessed by examining weight-shifting movements during spontaneous exploration using the cylinder test (Schallert et al., 2000). 6-OHDA lesions are also associated with sensory neglect of contralateral stimuli, as demonstrated by a variety of orientation tests such as whisker touch, snout probe and olfaction (Dunnett & Iversen, 1982). Contralateral neglect is also demonstrated using more complex tests such as operant tasks using the 9-hole box, where animals are required to respond to visual stimuli by initiating and executing correct nose-poke responses to either side of the head (Carli et al., 1985; 1989; Dowd & Dunnett, 2005).

Since the unilateral 6-OHDA-lesion model of PD displays a number of behavioural deficits that can be efficiently measured, this model allows the effective assessment of potential treatments, since any treatments that attenuate 6-OHDA lesion-induced behavioural deficits could prove to be clinically useful in providing symptomatic relief of Parkinson's disease.
1.3.2 Genetic models of Parkinson’s disease

The precise aetiology of Parkinson’s disease is unknown, however, underlying genetic causes have been identified in a small number of cases (see section 1.2.1). These genes have consequently been manipulated in vivo to create genetic animal models of Parkinson’s disease. This genetic modification of the genome is achieved in one of two ways: (1) by removing or altering a gene of interest so that it no longer functions (knock-out models), or (2) by adding a gene of interest to the genome so that it is expressed (transgenic models).

1.3.2.1 Gene Knock-out & Transgenic models of Parkinson disease

Since α-synuclein has been implicated in the pathophysiology of Parkinson’s disease and is a main constituent of Lewy bodies (a pathological hallmark of the disease), the α-synuclein gene has attracted particular interest. However, while α-synuclein knock-out mice have been developed, the genetically modified mice are viable, fertile, do not display any pathological features of Parkinson’s disease and do not exhibit alterations in dopaminergic pathways (Abeliovich et al., 2000). They do however show reduced striatal dopamine levels, attenuation of dopamine-dependent locomotor activity which is induced by amphetamine challenge and increased dopamine release following paired stimuli which suggests that α-synuclein negatively regulates dopamine neurotransmission (Abeliovich et al., 2000). Conversely, transgenic mouse models expressing wild-type human α-synuclein show a number of pathological features including the presence of α-synuclein and ubiquitin-positive intraneuronal inclusions in the neocortex, hippocampus and substantia nigra (Masliah et al., 2000). These inclusions however lack fibrillary aggregates which are typically seen in Lewy bodies (Orth & Tabrizi., 2003). In addition, TH-positive nerve terminals and TH levels in the striatum are markedly reduced and a significant deficit in motor performance is observed when compared to nontransgenic littermate controls (Masliah et al., 2000). In another study, transgenic mice expressing mutant and wild-type human α-synuclein also develop locomotor deficits, showed neuronal degeneration and exhibited Lewy body-like intraneuronal inclusions in some motor neurones (van der Putten et al., 2000). These studies indicate that higher expression of α-synuclein can result in its accumulation, aggregation and neurodegeneration, and highlight a central role of α-synuclein in the pathological process of both familial and the more frequent sporadic forms of Parkinson’s disease.
Although great focus has been placed on the α-synuclein gene other potential genetic models of Parkinson's disease have also been generated. These include the Parkin knock-out mice which show alterations in the extracellular dopamine levels in the striatum and deficits in behavioural tasks, but no loss of dopaminergic neurones in the substantia nigra (Goldberg et al., 2003), and the DJ-1 knock-out mice which show age-dependent and task-dependent motor deficits that are detectable by 5 months of age. While no degeneration of dopamine neurones in the substantia nigra is observed in this model, alterations in striatal dopamine levels, striatal dopamine release and re-uptake are observed suggesting that DJ-1 plays a role in nigrostriatal dopamine function (Chen et al., 2005).

Since the genetic models of Parkinson's provide information of gene function, these models are extremely valuable research tools and are of absolute importance so that we can further understand the genetic involvement in the pathogenesis of the disease. In addition, like the neurotoxin models, the genetic models of Parkinson's disease will also help in the development of new therapies since any potential treatments would need to correct the deficits seen in these models.

1.4 Neurotransplantation as a treatment for Parkinson disease

Graft-induced functional recovery of behavioural deficits induced by striatal dopamine depletion was first described in the late 70's when pieces of embryonic dopamine tissue, which were grafted into unilateral lesioned rats, showed attenuation of drug-induced rotational asymmetry (Perlow et al., 1979; Björklund et al., 1979). Since then improvements in a number of behavioural impairments have been shown following intrastriatal grafting of dopamine neurones. The success of neurotransplantation in rodent and monkey models has consequently resulted in clinical trials of neurotransplantation in an attempt to provide an effective, long-term treatment for Parkinson's disease.

1.4.1 Functional recovery of lesion-induced behavioural deficits

Transplantation of embryonic mesencephalic dopamine neurones into the striatum of unilateral depleted rodents and monkeys has provided evidence that grafted dopamine neurones can survive, integrate and improve lesion-induced motor deficits such as
drug-induced rotational bias (Abrous et al., 1993; Perlow et al., 1979; Björklund & Stenevi., 1979; Dunnett et al., 1983; Annett et al., 1994). Interestingly, drug-induced rotational asymmetry has not only been significantly reduced, but in some cases, grafts of dopamine neurones have resulted in overcompensation of rotational bias (Björklund et al., 1980; Herman et al., 1985). Grafts of dopamine neurones have also shown improvements in other lesion-induced deficits such as contralateral sensory neglect (Dunnett et al., 1987), hypokinesia (Dunnett et al., 1981) and learning deficits (Dowd & Dunnett, 2004). Full recovery from all behavioural deficits is however not observed, with grafts unable to improve some behaviour impairments including skilled paw use and limb preference (Dunnett et al., 1987; Annett et al., 1994). This incomplete recovery may be attributed to partial restoration of striatal dopamine levels (Björklund & Dunnett., 1983), and incomplete reinstatement of striatal dopamine synthesis levels (Björklund & Dunnett., 1983), which are a result of poor survival of transplanted dopamine neurones (Björklund., 1992). Alternatively, absence of complete recovery of behavioural deficits could be a result of graft placement, since the location of grafts within the denervated striatum has shown to determine the type of deficit which is restored (Björklund et al., 1980; Dunnett et al., 1981).

1.4.2 Dopamine grafts - mechanism of function

The mechanisms by which grafted dopamine neurones have their effects are unclear, however, evidence suggests that functional recovery is not solely due to symptomatic relief via ‘pharmacological action’ of grafted dopamine neurones, but is also associated with disease recovery via graft integration and resultant cellular changes (Björklund., 1992). This is particularly highlighted by the effects of dopamine delivery therapies. Polymer capsules that gradually release dopamine have been transplanted into the unilateral dopamine-depleted striatum and have shown to reduce apomorphine-induced rotations (Becker et al., 1990; Winn et al., 1989). However, while these treatments have shown to improve some behavioural deficits, they are unable to improve rotational behaviour induced by amphetamine stimulation, and therefore cannot account for the whole range of functional recovery which is observed following transplantation of dopamine neurones (Dunnett & Björklund., 1994). Dopamine grafts must therefore function by integrating with the host striatum in addition to having a pharmacological mechanism of action. Grafted dopamine
neurones have been shown to integrate with the host striatum by growing extensive axonal projections which innervate the denervated striatum by forming specific synaptic contacts with host striatal medium spiny projection neurones (Freund et al., 1985). Some of these grafted neurones have shown similar electrophysiological firing properties to those neurones found in the intact substantia nigra, and have been shown to respond to stimulation of the cortex and the striatum (Fisher et al., 1991), indicating functional integration with host neurones. Grafted dopamine neurones have also been shown to correct cellular deficits caused by striatal dopamine loss. For instance, supersensitivity of postsynaptic D1 & D2 dopamine receptors which occurs following striatal denervation is normalised following grafts of dopamine neurones (Savasta et al., 1992; Rioux et al., 1991; Dawson et al., 1991), as are levels of dopamine receptor-induced expression of the protein product c-fos (Cenci et al., 1992), which have been shown to increase following apomorphine stimulation of postsynaptic dopamine receptors and to decrease in response to amphetamine-induced presynaptic dopamine release in the denervated striatum (Cenci et al., 1992). Further evidence for normalisation of striatal function has been shown by the reinstatement of normal striatal peptide levels. Levels of enkephalin and enkephalin mRNA in striatopallidal projection neurones and levels of the GABA synthesising enzyme, GAD and expression of its mRNA in GABAergic striatal neurones are substantially increased following dopamine depletion of the striatum (Gerfen., 1992; Manier et al., 1991; Mendez et al., 1993; Segovia et al., 1990). These levels are subsequently restored to normal levels, or are partially reduced following dopaminergic cell transplantation (Sirinathsinghji & Dunnett., 1991; Cadet et al., 1991; Manier et al., 1991; Mendez et al., 1993; Segovia et al., 1989; 1991), thus demonstrating the ability of dopamine grafts to reinstate normal balance of striatal output activity.

1.4.3 Neurotransplantation - Clinical Trials

The functional benefits observed in animal models following intrastriatal grafts of dopamine neurones have resulted in a series of clinical trials where Parkinson's disease patients have received intrastriatal grafts of mesencephalic tissue from aborted foetuses. Although results from these trials have not shown the complete reversal of disease symptoms, moderate improvement in a number of motor deficits including rigidity, hypokinesia and bradykinesia have been observed (for reviews see Olanow et
al., 1996; Lindvall, 1997 and references Wenning et al., 1997; Lindvall et al., 1990; 1992; Kopyov et al., 1997; Peschanski et al., 1994; Freed et al., 1992). There are also improvements in on-going L-dopa treatment, with a reduction in time spent in 'off' phase (where L-dopa does not provide symptomatic relief) and an increase in the duration of positive L-dopa effects following a single dose (Freeman et al., 1995; Lindvall et al., 1994; Wenning et al., 1997). In some cases, grafts of dopamine neurones have resulted in L-dopa treatment being reduced (Wenning et al., 1997; Freed et al., 1992) and in at least one case, L-dopa treatment has been withdrawn completely (Lindvall et al., 1994). Confirmation of graft function is demonstrated using positron emission tomography (PET), which shows increased uptake of \[^{18}\text{F}\]fluorodopa in the grafted striatum (Lindvall et al., 1990; 1994; Wenning et al., 1997; Kordower et al., 1995; Remy et al., 1995). Direct evidence of graft-induced clinical improvements has also come from the histological examination of the post-mortem brain. Two patients, which displayed marked motor improvements and increased \[^{18}\text{F}\]fluorodopa uptake following transplantation, who died over a year after transplantation from causes unrelated to surgery, showed the presence of large grafts, which contained thousands of dopamine neurones that extensively reinnervated the striatum (Kordower et al., 1995; 1998). The clinical benefits seen in some patients are also long-term as shown by sustained motor improvements and \[^{18}\text{F}\]fluorodopa uptake years following transplantation (Lindvall, 1997; Lindvall et al., 1994; Dunnett et al., 2001).

Although these studies show that neurotransplantation can be effective for at least some patients, all of these clinical trials have used open-labelled studies (where there is no control group and both patient and physician are aware that transplant has been received) and are therefore subject to being influenced by placebo effects and physician bias (Freeman et al., 1999). To overcome these issues, two of the most recent transplantation studies have used randomised, double-blind, placebo-controlled trials, where patients either received grafts of dopamine neurones or sham surgery (burr holes were drilled but no tissue was implanted), with the patient and physician unaware of what procedure had been received (Freed et al., 2001; Olanow et al., 2003). Interestingly, although \[^{18}\text{F}\]fluorodopa uptake in these two studies provided evidence of graft survival and function, in the study by Freed, moderate improvements were only reported in patients under the age of 60 years 12 months after transplantation, and in the study by Olanow, no clinical benefit whatsoever was
observed after 2 years. Both studies did however report the development of dyskinesia in a proportion of patients, the severity of which was much worse than that which has been reported in open-labelled trials.

Although these results raise considerable concerns about the readiness of cell replacement strategies for the clinical treatment of Parkinson's disease, it is important to note that the transplantation methods used in these studies differed from the open-labelled trials, and could therefore account for the discrepancies in the results (Dunnett et al., 2001; Lang & Obeso., 2004; Winkler et al., 2005). In the study by Freed, less tissue was transplanted compared to most open-labelled trials with only 2 donors used per transplant, tissue was stored for up to 4 weeks prior to transplantation and was implanted as a single concentrated deposit in the neostriatum using an unvalidated 'noodle' preparation. Furthermore, no immunosuppression was used (Dunnett et al., 2001; Winkler et al., 2005).

With the study by Olanow, tissue was grafted as solid pieces, unlike most of the open-labelled trials, which used cell suspensions, and although immunosuppression treatment was given, only cyclosporine was used and not the triple immunosuppressive treatment (cyclosporine, azathioprine & prednisolone) used in previous studies. In addition, cyclosporine was discontinued after 6 months, despite patients being followed for 2 years – a level of immunosuppression which is probably insufficient to block early rejection of grafted dopamine neurones before they have had a chance to become fully established (Winkler et al., 2005).

The negative effects of these different methodologies on graft function and survival are clearly demonstrated. For instance, in the study by Freed, histological examination of the brains of two patients who died months following transplantation surgery, revealed the number of surviving dopamine neurones contained in the grafts were less than half of what had been reported in previous open-labelled trials; 7000-40000 cells in double-blind trial vs. 80000-135000 cells in open-labelled trials (Dunnett et al., 2001; Winkler et al., 2005). In addition, in the study by Olanow, patients grafted with tissue from 4 donors showed improvements up to 6 months after surgery, but then deteriorated back to pre-graft values after 18 months following transplantation. This deterioration might reflect a slow rejection process associated with incomplete immunosuppression.
Despite the negative results from the double-blind, placebo-controlled studies, transplantation is still an effective strategy for the treatment of PD, but it is clear from these results that certain cell preparation, storage and transplantation techniques need to be refined before further clinical trials are attempted. All studies should also assess the clinical effects of transplantation using the core assessment program for intracerebral transplantation (CAPIT) protocol, which would allow studies to be compared. In addition, use of tissue from aborted foetuses in transplantation studies is complicated because of a combination of ethical issues, logistical problems, and the amount of tissue required per transplant (Björklund & Lindvall., 2000). Transplantation strategies will therefore, only provide a practical treatment for PD if these supply issues can be resolved, and if a suitable replacement tissue source can be found.

Tissue containing catecholamine secreting cells such as adrenal chromaffin cells or cells of the carotid body may be possible alternatives. Adrenal grafts transplanted into either the lateral ventricles or the striatum have shown reduction in behavioural deficits induced by unilateral 6-OHDA lesions in rats (Becker & Freed., 1988; Freed et al., 1990; Borlongan et al., 1998; Date., 1996). Similarly, glomus cells of the carotid body, which normally release dopamine in response to hypoxic environments, have also been shown to alleviate behavioural deficits in dopamine depleted rats and monkeys following intrastratal transplantation (Luquin et al., 1999; Espejo et al., 1998; Toledo-Aral et al., 2002). However, an extensive series of clinical studies during the 80’s and 90’s failed to find good survival and functional effect of such peripheral neuroendocrine autografts. Alternatively, replacement dopamine neurones could come from the use of stem cells since these cells have the ability to generate dopamine neurones under certain environmental conditions.

1.5 Stem cells
The ability of stem cells to generate cells of the central nervous system has resulted in a substantial amount of interest over the past decade, mainly because of their potential use in cell replacement therapies for neurodegenerative disease. This section gives a brief description of stem cell characterisation, origin and function before discussing the potential use of embryonic, foetal and adult neural stem cells in neural transplantation. The plasticity of multipotent stem cells and their ability to trans-
differentiate into cells of the nervous system will also be discussed in terms of their use as a donor source for cell replacement strategies.

The terminology used to describe stem cells and their progeny varies from person to person and can become quite complicated. In this thesis I adopt a relatively inclusive definition. The term *stem cell* is used to describe a cell with multipotentiality and the capacity for self-renewal. *Progenitor cells* have limited self-renewal abilities and have uni- or multipotentiality, and the term *precursor cell* is used to describe either mixed or unknown proliferating populations (Weiss *et al*., 1996; Seaberg & van er Kooy., 2002). For simplicity, in this thesis I will only use the term stem cell when describing cells that are definitely stem cells, and in all other cases I will use the term, progenitor cell.

### 1.5.1 What are stem cells?

Many definitions of stem cells exist, but they are functionally defined as cells which have the ability to self-renew and generate differentiated progeny (Watt & Hogan., 2000; Morrison *et al*., 1997; Temple., 2001). This self-renewal can be achieved by either symmetric division, where the two daughter cells are stem cells, or by asymmetric division, where one daughter cell is a stem cell and the other is a restricted progenitor cell. These progenitor cells have limited self-renewal capacities before terminally differentiating into multiple, yet distinct cell lineages (Watt & Hogan., 2000; Morrison *et al*., 1997).

Stem cells are prevalent in organs and tissues throughout the entire developing and adult organism, such as the heart (Beltrami *et al*., 2003; Urbanek *et al*., 2003), skin (Toma *et al*., 2001; Blanpain *et al*., 2004) and even in the central nervous system (McKay., 1997; Gage *et al*., 1995), where their discovery in the adult has overturned the long-standing dogma that the brain is a structure incapable of regeneration. Their function in development is to proliferate and generate cells enabling tissues and organs to mature, and in the adult, it is believed they participate in tissue homeostasis by replacing differentiated cells lost to physiological turnover or injury (Fuchs & Serge., 2000; van der Kooy & Weiss., 2000; Weiss *et al*., 1996).

The classification of stem cells is based on their lineage potentials, with the zygote an example of a *totipotent* stem cell since it gives rise to all cells that constitute a multicellular organism. From this totipotent state, generation of cells with more restricted differentiation capacities are formed. Embryonic stem cells are derived from
the inner cell mass of the developing blastocyst and can differentiate into ectodermal, endodermal and mesodermal derivatives (Kim et al., 2003, see section 1.5.2), but not into cells of the extraembryonic membranes that form cells of the placenta (Gage., 2000). Embryonic stem cells are therefore slightly more restricted than totipotent cells and are thus referred to as pluripotent stem cells. Specialised cells restricted to forming cell phenotypes of a particular lineage are known as multipotent stem cells and are defined by the organ from which they are derived. Neural stem cells are examples of multipotent stem cells and are derived from the nervous system. These multipotent cells have the ability to produce lineage-restricted daughter cells called neural progenitor cells, which generate neurones, astrocytes and oligodendrocytes (for a review see Gage., 2000).

The ability to generate neurones from stem cells has provided a unique source of cells for the treatment of neurodegenerative disorders via neural transplantation strategies. However, for stem cells to be successful in the treatment of Parkinson’s disease they need to be directed not only into dopamine phenotypes, but also into dopamine projection neurones. Here we consider the possible stem cell sources that may be useful in the generation of dopamine neurones and the extent to which directed dopamine differentiation has been achieved.

1.5.2 Embryonic stem cells

Embryonic stem (ES) cells are undifferentiated, pluripotent cells derived from the inner cell mass of the blastocyst. Generated initially from embryos of mice (Evans & Kaufman., 1981; Martin., 1981), ES cells have now been successfully developed from monkey blastocysts (Thomson et al., 1995) and from human embryos acquired from patients undergoing in vitro fertilisation (IVF) treatment (Thomson et al., 1998; Reubinoff et al., 2000; Schuldiner et al., 2001). Like mouse ES cells (Evans & Kaufman., 1981; Martin., 1981), human ES cells can undergo unlimited in vitro proliferation whilst remaining in an undifferentiated state. Their pluripotent properties are also retained following proliferation, as shown by the injection of these cells into immuno-deficient SCID mice and the subsequent formation of teratomas, which, by definition contain cells derived from all three germ layers of the developing embryo, including cells of the nervous system (Thomson et al., 1998; Reubinoff et al., 2000).
1.5.2.1 Neural differentiation of ES cells

The potential of human ES cells to generate neural phenotypes, coupled with their ability to proliferate indefinitely could essentially provide a good, continuous source of neurones for transplantation. However, while human ES cells have been shown to behave like endogenous progenitors and generate olfactory bulb neurones following transplantation into the subventricular zone of rats (Tabar et al., 2005), the necessary requirements to generate sufficient quantities of suitable neurones from these cells are unknown and are currently being explored. One approach to induce ES cell neural differentiation has been through the manipulation of the culture environment. Retinoic acid, a developmental factor involved in anterior-posterior patterning of the neural tube, has been shown to be a potent neural-inducing factor (Takahashi et al., 1999) and its addition to mouse ES cells has resulted in the generation of large numbers of neurones (Bain et al., 1995; Fraichard et al., 1995). The addition of basic fibroblast growth factor (bFGF) (fibroblast growth factors are essential molecules involved in the development and regulation of cell proliferation, migration and differentiation) to proliferating mouse ES cells, and their subsequent differentiation following bFGF withdrawal has also been shown to promote neural differentiation, with over 60% of total cells expressing the neuronal marker MAP2, and up to 15% of total cells expressing the glial cell marker GFAP (Okabe et al., 1996). Furthermore, retinoic acid and β nerve growth factor (βNGF) have been shown to influence differentiation of human ES cells into neural phenotypes (Schuldiner et al., 2001). Interestingly, these neurones not only stain for neurofilament heavy-chain protein which is found in mature neurones, but they also express serotonin or dopamine receptors in addition to expressing dopa decarboxylase, which is an enzyme involved in the synthesis of these neurotransmitters (Schuldiner et al., 2001), thus indicating that these neurones may indeed be dopaminergic. The generation of dopaminergic neurones is essential if neural transplantation is going to be a successful treatment for Parkinson's disease and so conditions that promote dopaminergic differentiation need to be defined.

Recently, the expansion of mouse ES cells in media deficient in HEPES buffer but supplemented with bFGF, sonic hedgehog (a developmental factor involved in the dorsal-ventral patterning of the neural tube) and fibroblast growth factor 8 (FGF8), followed by their withdrawal and addition of ascorbic acid, enhanced neural differentiation, with over 30% of neurones being immunopositive for tyrosine.
hydroxylase (TH) (Lee et al., 2000). Mouse ES cells have also been shown to differentiate into dopamine neurones following their culture in media containing bFGF and epidermal growth factor (EGF) and differentiation in media consisting of a host of survival-promoting factors (Rolletschek et al., 2001). The addition of these survival-promoting factors showed increased gene expression of TH and NURR1, as well as increasing the percentage of neurones immunopositive for TH from 22% to 43%.

Nurrl is a transcription factor specific to midbrain dopamine neurones alone (Zetterström et al., 1997) and mouse ES cells genetically modified to express this gene have shown a 4-5 fold increase in dopaminergic neurone expression (Chung et al., 2002). Exposing these cells to the conditions used by Lee and colleagues (see above) further increased dopamine differentiation, with 62% of neurones staining immunopositive for TH (Chung et al., 2002). Although TH is an enzyme involved in dopamine synthesis, one needs to be cautious when using it to label dopamine neurones since the enzyme is also involved in the synthesis of other neurotransmitters such as serotonin. Consequently neurones expressing TH are not necessarily dopaminergic. However, in all three of these studies the absence of double labelled cells following double immunohistochemistry for TH and serotonin indicated that these neurones were indeed of dopaminergic phenotypes.

An alternative approach to inducing dopamine neurones is by the use of feeder layers. Mouse ES cells co-cultured with stromal feeder cells have shown enhanced dopamine neurone generation, with 30% of neurones staining positive for TH (Kawasaki et al., 2000). When grafted into the striatum of unilateral 6-OHDA lesioned mice, the survival rate of dopamine neurones was shown to be 22%, quite impressive considering the survival of embryonic mesencephalic neurones is in the range of 3-10% (Olanow et al., 1996; Dunnett., 1992; Roybon et al., 2004). However, while survival was good, no examination of lesion-induced behavioural deficits or its subsequent recovery following grafting was reported, so whether or not these neurones were functional remains unknown.

1.5.2.2 Functionality of ES-derived dopamine neurones

Over the past few years, great progress has been made in generating neurones of a dopaminergic phenotype. Demonstrating their functionality is however, imperative if
ES cells are going to be a serious candidate as a donor source for cell therapy in PD. Until a few years ago, such evidence was lacking, but two reports published in 2002 have shown functional recovery of 6-OHDA lesion-induced behavioural deficits following grafts of ES-derived dopamine neurones. The first of these two papers grafted a low concentration of ES cells into the rat striatum, arguing that a low concentration decreases ES cell-cell contact and thus increases the influence of the host striatum environment on these cells (Björklund et al., 2002). The second used mouse Nurrl ES cell lines (generated by the insertion of the rat Nurrl cDNA fragment) and expanded them in the conditions defined by Lee and colleagues (see above) (Kim et al., 2002). Following transplantation of these cells into the striatum of unilateral dopamine depleted rats, both groups reported significant reductions in amphetamine-induced rotational bias several weeks post-transplantation. Significant improvements in a variety of non-pharmacological behavioural tests such as adjusting step, cylinder and paw reaching tests were also observed and electrophysiological recordings of grafted dopamine neurones showed similar characteristics to endogenous mesencephalic neurones (Kim et al., 2002). Non-behavioural tests were also used in the study by Björklund to further confirm the functionality of their grafted dopamine neurones. Using positron emission tomography (PET) and carbon-11-labeled 2β-carbethoxy-3β-(4-fluorophenyl) tropane ([11C]CFT), which is a dopamine transporter ligand, showed substantial reinnervation of the striatum. In parallel, functional magnetic resonance imaging (fMRI) showed restoration of appropriate haemodynamic changes in the striatum following amphetamine challenge. Collectively, these results provide encouraging evidence for the potential use of ES cells in cell replacement therapy for PD. The use of human ES cells is however, controversial and is associated with a number of ethical issues. The main ethical debate surrounds the moral status of the human embryo, with many people believing that the human embryo is equivalent to a human being. Therefore, destroying human embryos to obtain stem cells for research or disease treatment is seen as illegal (since it’s equivalent to murder) and immoral (since killing one person to save or treat the life of another person is unjustifiable) (McLaren., 2001; Robertson., 2001; Roche & Grodrin; 2000). There are also some safety concerns regarding the use of these stem cells, for instance the potential for mutations is always a concern for cultured cells (Svendsen & Smith., 1999), and any grafted undifferentiated cells will have the ability
to divide indefinitely \textit{in vivo} resulting in the formation of tumours, as seen in five animals in the study by Björklund (Björklund \textit{et al.}, 2002) and more recently in the study by Thinyane and colleagues (Thinyane \textit{et al.}, 2005). This is particularly problematic since not all ES cells undergo differentiation, and therefore a rigorous selection process is required to remove or eliminate such undifferentiated cells. Although efforts have been made to eliminate the presence of undifferentiated cells by introducing suicide genes (Schuldiner \textit{et al.}, 2003; Fareed & Moolten., 2002) all these issues need to be fully addressed before ES cells enter clinical trials. On a separate issue, introducing genes into human embryos has raised great concern, since such techniques could open the door to creating offspring with certain desirable characteristics (Robertson., 2001).

\subsection*{1.5.3 Neural stem cells}

Neural stem cells are multipotent cells capable of generating neurones, astrocytes and oligodendrocytes. They can be isolated from the developing or adult brain and cultured in the presence of certain mitogens such as epidermal growth factor (EGF) (Reynolds and Weiss., 1992; Winkler \textit{et al.}, 1998) or basic fibroblast growth factor (bFGF) (Qian \textit{et al.}, 1997; 2000). These cells can proliferate as monolayer cultures or form free-floating aggregates of undifferentiated cells called \textit{neurospheres} (Reynolds & Weiss., 1992), and differentiate into neural phenotypes following mitogen withdrawal, the addition of serum and exposure to suitable substrates. The presence of specific mitogens in the culture environment is essential for cell expansion, however experimental evidence suggests that EGF and bFGF act on different progenitor populations, with EGF favouring proliferation of glial progenitors and bFGF favouring the proliferation of neuronal progenitors (McKay., 1997; Kuhn \textit{et al.}, 1997; Kilpatrick & Bartlett., 1995). In addition to the \textit{in vitro} manipulation of these cells, neural stem cells can also be transplanted into the intact or lesioned brain where they show the ability to survive, migrate and differentiate into cells of the nervous system (Gage \textit{et al.}, 1995b; Minger \textit{et al.}, 1996; Fricker \textit{et al.}, 1999; Englund \textit{et al.}, 2002).

\subsubsection*{1.5.3.1 Foetal neural stem cells}

Foetal neural stem cells can be isolated from various regions of the developing brain, including (but not restricted to) the ventral mesencephalon (VM), the cortex (CTX)
and from the whole (WGE), lateral (LGE) and medial (MGE) segments of the ganglionic eminence (Smith et al., 2003). Stem cells from these areas have been isolated at a variety of ages (see chapter three), and from a range of species including mice (Murphy et al., 1990; Reynolds & Weiss., 1992), rats (Smith et al., 2003; Rosser et al., 1997; Carvey et al., 2001), and pigs (Armstrong et al., 2002, 2003). Similarly, the isolation, proliferation and neural differentiation of human neural stem cells has also been achieved (Svendsen et al., 1998; Carpenter et al., 1999; Caldwell et al., 2001), and has generated particular interest due to their potential clinical use in PD. However, while these cells have demonstrated their ability to spontaneously differentiate into neurones, the generation of dopamine neurones is typically low and decreases significantly with cell expansion (Yan et al., 2001). Enhancing dopamine differentiation is therefore crucial if these cells are going to provide a source of cells for transplantation. However, whilst extensive research has focused on this area, the differentiation of neural stem cells into dopamine phenotypes is still proving difficult.

1.5.3.2 Enhancing dopamine differentiation in vitro

One approach to enhance dopamine differentiation of neural stem cells is by the use of genetically immortalised neural stem cells, which overexpress the transcription factor Nurrl. This factor has been shown to be essential for the induction of midbrain dopamine neurones, which fail to develop in Nurrl-depleted mice (Zetterström et al., 1997), due to inadequate neural survival and the inability of mesencephalic neurones to terminally differentiate into dopaminergic phenotypes (Saucedo-Cardenas et al., 1998). Although differentiation of these immortalised cells does not generate neurones (Wagner et al., 1999; Kim et al., 2003), their co-culture with primary E16 VM cells and specifically with ventral mesencephalic type 1 astrocytes results in a substantial increase in the number of TH positive neurones, with over 80% of cells staining immunopositive for TH (Wagner et al., 1999). Interestingly, these TH positive neurones also release dopamine upon depolarisation and they also express midbrain markers. Similarly, enhanced TH expression is observed following coculturing of Nurrl-overexpressing neural progenitor cells with primary astrocytes derived from human foetal brains (Kim et al., 2003). In addition, this dopaminergic differentiation can be further enhanced by culturing Nurrl-overexpressing cells in the presence of sonic hedgehog (SHH) and FGF8 (Kim et al., 2003), which are
developmental signals of midbrain dopamine neurones and have previously shown to promote ventral midbrain fates in rat neural plate explants (Ye et al., 2003). The combination of Nurr1-overexpressing neural progenitor cells, SHH and FGF8 result in more than 90% of neural progenitor cells expressing TH, therefore indicating that sonic hedgehog and FGF8 are potent inducers of dopaminergic phenotypes.

Alternatively, enhanced dopamine differentiation of neural progenitor cells has been achieved by the addition of a variety of factors into the culture environment. For instance, the differentiation of bFGF-expanded human foetal VM tissue shows 64-69% of cells expressing the neuronal marker MAP2 and about 12% of cells immunopositive for TH. However, following differentiation in media supplemented with dopamine (10μM), forskolin (10μM) and 50ng/ml brain derived neurotrophic factor (BDNF), TH expression is significantly enhanced, with over 60% of cells expressing TH (Riaz et al., 2002). The addition of extrinsic cytokines to the differentiation media has also been shown to increase dopamine differentiation. Rat E14.5 VM cells expanded in media containing EGF and differentiated in serum containing media supplemented with interleukin-1 (IL-1), showed increased TH expression in developing neurones (Ling et al., 1998). Further differentiation in media containing 10% serum, IL-1, interleukin-11 (IL-11), leukemia inhibitory factor (LIF) and glial cell line-derived neurotrophic factor (GDNF) not only enhanced the development of TH cells, but also further increased TH expression, with 25% of all cells expressing TH. Interestingly, the addition of conditioned media from striatal cell cultures and fragments of mesencephalic membranes to the cocktail of cytokines resulted in TH neurones acquiring more mature morphologies, which included large somas and numerous branching processes with varicosities (Ling et al., 1998). Using the same differentiation protocol, human ventral mesencephalic progenitors have also been demonstrated to generate mature neurones with dopaminergic phenotypes, which release dopamine and express the dopamine transporter protein (DAT) (Storch et al., 2001). In this study, long-term EGF & bFGF-mediated expansion of ventral mesencephalic cells was only achieved following proliferation in 3% atmospheric oxygen, a concentration representative of normal interstitial O2 levels, instead of the standard 20% O2. The importance of O2 levels on proliferation and differentiation of neural progenitor cells has also been reported elsewhere. The expansion E12 rat VM bFGF-responsive progenitor cells in 3% atmospheric oxygen resulted in increased cell
expansion compared to cells grown in standard (20%) oxygen environments (Studer et al., 2000). This increased expansion was as a combined result of increased cell proliferation and decreased cell death. Following 5 days differentiation, when cells exposed to lower oxygen levels were compared to cells grown in standard conditions, an increase in neurogenesis (73% vs. 63%) and an increase in dopamine differentiation (56% vs. 18%) was observed (Studer et al., 2000). The detrimental effects of high oxygen environments may be mediated through oxidative stress, a process which has been suggested to contribute to the death of substantia nigra dopamine neurones in PD (Sian et al., 1994; Olanow., 1993; Fahn & Cohen., 1992; Olanow & Tatton., 1999). Indeed, neuroprotective effects of anti-oxidants on dopamine neurones have been demonstrated by a number of studies (see chapter four).

The antioxidant N-acetylcysteine has shown to increase dopamine differentiation of E14 rat VM cells expanded in EGF (Rodrigues-Pallares et al., 2001), and lazaroids, which are compounds that prevent lipid peroxidation and oxidative stress, have been shown to increase the survival of dopamine neurones in primary E14 rat VM cultures (Frodl et al., 1994). Interestingly, the anti-oxidant ascorbic acid has also been shown to increase dopaminergic neurone differentiation of expanded rat E12 VM progenitor cells (Yan et al., 2001). However, rather than being a neuroprotective agent and defending against oxidative stress, the authors of this study suggest that ascorbic acid may increase dopamine expression by activating dopamine differentiation pathways.

1.5.3.3 Transplantation of expanded neural progenitor cells

Although a considerable number of studies have reported manipulation of progenitor cells in vitro, the number of reports assessing the behaviour of expanded neural progenitor cells following transplantation is relatively scarce. However, results from this limited number suggest that grafted progenitor cells can survive for long periods of time, migrate extensively and differentiate into neuronal and glial phenotypes. These characteristics have been shown following transplantation of bFGF-expanded rat basal forebrain progenitor cells into various regions of the adult rat brain. Grafted cells are apparent 7 months post-transplantation and differentiation into neuronal and glial cells is observed (Minger et al., 1996). Similarly, transplants of long-term expanded human neural progenitor cells into the striatum and neurogenic regions of the neonate brain show differentiation into neural phenotypes, extensive migration...
and cell survival for up to 1 year post-transplantation (Englund et al., 2002). Interestingly, when transplanted into the adult brain these neural progenitor cells are influenced by local signals to migrate and differentiate into specific cell types (Fricker et al., 1999; Englund et al., 2002), thus indicating the sensitivity of these cells to the environment in which they are placed.

1.5.3.4 Dopamine differentiation of transplanted neural progenitor cells

The generation of dopamine neurones from expanded neural progenitor cells following transplantation is currently proving extremely difficult. A number of studies have attempted to address this issue but have generally yielded disappointing results. Unlike transplantation of primary dopamine neurones, which show large grafts and the attenuation of behavioural deficits in animal models of PD, EGF-expanded human and rat ventral mesencephalon progenitors grafted into 6-OHDA lesioned rats form small grafts, show limited cell migration, few surviving dopamine neurones and no functional recovery of lesion-induced behavioural deficits (Svendsen et al., 1996). Porcine EGF and bFGF-expanded VM progenitors transplanted into the 6-OHDA lesioned rat generated large surviving grafts 9 weeks post-transplantation, however little dopaminergic differentiation was observed and again, no improvements in rotational bias were evident (Armstrong et al., 2002; 2003). Slight progress has been achieved following expansion of human VM progenitors in a combination of EGF & bFGF before their subsequent transplantation into the dopamine depleted striatum of rats. At 2 weeks post-transplantation, large grafts were observed indicating good cell survival, however by 20 weeks post-transplantation, graft size was markedly reduced, the grafts contained few cells, and a large number of astrocytes where observed migrating away from the graft into the host striatum. Importantly, in this study partial reduction in amphetamine-induced rotational bias was observed in 2 animals and analysis of the grafts showed the presence of numerous TH positive neurones in these two cases (Svendsen et al., 1997a). Although encouraging, results are inconsistent and when compared to grafts of primary tissue, dopamine differentiation and cell survival is less than impressive.

To date, the most promising results have been observed by differentiating cells prior to transplantation. In a study by Studer et al., (1998), rat VM cells were expanded with bFGF for 7 days and then differentiated them as free floating aggregates in
roller-tube cultures with serum containing media. Following 7 days differentiation cells were transplanted as aggregates into 6-OHDA lesioned rats. 3 months post-transplantation, grafts were reportedly of similar size to those found after primary cell transplantation and contained over 1200 TH positive neurones. These TH neurones significantly reduced amphetamine-induced rotations in 5 out of 7 grafted rats, thus demonstrating their functionality (Studer et al., 1998). Reduction in amphetamine-induced rotational bias was also observed following transplantation of clonal cell lines derived from E14.5 rat VM cells (Carvey et al., 2001). These cells were pre-differentiated for 5 hours in media containing 10% serum, interleukin-1 (IL-1), interleukin-11 (IL-11), leukemia inhibitory factor (LIF) and glial cell line-derived neurotrophic factor (GDNF) prior to transplantation in 6-OHDA lesioned rats. An 88% reduction in rotational bias was observed 8 weeks post-transplantation and grafts contained numerous dopamine neurones. However, when compared to grafts of primary VM tissue, significantly less dopamine neurones were observed, and importantly, the survival rate of grafted dopamine neurones was low (Carvey et al., 2001).

The pre-differentiation procedures used in these studies seem logical since instructive signals that influence dopaminergic differentiation seem to be absent from the striatum (Tang et al., 2002). However, while the differentiation of expanded neural progenitors into functional dopamine neurones is extremely encouraging, the survival rates of grafted dopamine neurones demonstrated in these studies are inadequate. For neural progenitors to be considered a valid source of cells in cell replacement therapies, not only do sufficient numbers of functional dopamine neurones need to be generated from cells undergoing longer expansion periods, but also, the survival of dopamine neurones following transplantation needs to be enhanced. Only when these issues are addressed will these cells be valuable in clinical trials.

1.5.4 Adult neural stem cells

The belief that the adult brain is a structure incapable of regenerating has now been proved wrong, with evidence showing the existence of continued neurogenesis in specific areas of the adult brain. Neurogenesis has been reported in a variety of brain areas (Gritti et al., 2002; Gould et al., 1999; Zhao et al., 2003), but predominantly occurs in two neurogenic regions, namely the dentate gyrus of the hippocampus and
the subventricular zone (SVZ) of the lateral ventricles (Altman & Das., 1965; Gage et al., 1995; Kuhn & Svendsen., 1999; Seaberg & van der Kooy., 2002). The generation of new neurones in the dentate gyrus was first revealed over 40 years ago using \(^3\)H-thymidine autoradiography (Altman & Das., 1965), and it has been reported that cells from this region migrate from the subgranular layer of the dentate gyrus into the granular cell layer where they differentiate into hippocampal granule cell neurones (Altman & Das., 1965; Kuhn et al., 1996). In the SVZ, neural stem cells are continuously generated and migrate along the rostral migratory stream into the olfactory bulb where they differentiate into dopaminergic and GABAergic interneurones (Lois & Alvarez-Buylla., 1993). Multipotent adult neural stem cells can be isolated from the central nervous system, proliferated in culture and differentiated into a host of neural phenotypes. This procedure is now well established with cells from rodents (Reynolds & Weiss., 1992; Richards et al., 1992; Gritti et al., 1996), monkeys (Gould et al. 2001), humans (Roy et al., 2000a; 2000b; Arsenijevic et al., 2001) and even post-mortem human brain (Palmer et al., 1995; 1997), however, cells dissected specifically from the dentate gyrus have been shown to have limited self-renewal capacities and unipotent characteristics, therefore indicating that these cells represent a population of restricted progenitors rather than stem cells (Seaberg & van der Kooy., 2002). The precise location of SVZ stem cells is also an area currently causing considerable debate. It has been suggested that these stem cells reside in the ependymal layer that line the ventricle walls, since when isolated, these cells are capable of self-renewal and generating neurones, astrocytes and oligodendrocytes (Johansson et al., 1999). Other reports however, indicate that the

In the absence of specific stem cell markers, the characteristics used to define stem cells \textit{in vitro} are based on the ability of cells to self-renew and show multipotent properties (Reynold & Weiss., 1992; 1996; Gage et al., 1995a; 2000). Although these features have been demonstrated for cells isolated from the SVZ (Gritti et al., 2002), there is some controversy over the self-renewal and multipotency of hippocampal cells. Cells isolated from the hippocampus have shown long-term self-renewal and the ability to differentiate into a range of neural phenotypes (Palmer et al., 1995; 1997), however, cells dissected specifically from the dentate gyrus have been shown to have limited self-renewal capacities and unipotent characteristics, therefore indicating that these cells represent a population of restricted progenitors rather than stem cells (Seaberg & van der Kooy., 2002). The precise location of SVZ stem cells is also an area currently causing considerable debate. It has been suggested that these stem cells reside in the ependymal layer that line the ventricle walls, since when isolated, these cells are capable of self-renewal and generating neurones, astrocytes and oligodendrocytes (Johansson et al., 1999). Other reports however, indicate that the
stem cells originate from subependymal layer, which lies adjacent to the ependyma. Evidence for this has been demonstrated following specific isolation of ependymal and subependymal cells and their subsequent in vitro proliferation and differentiation. These studies show that although ependymal cells can generate neurospheres in culture, they are unable to generate secondary neurospheres thus indicating their inability to self-renew (Chiasson et al., 1999; Morshead & van der Kooy., 2001). Furthermore, ependymal cells only have unipotent potentials since they can only differentiate into glial phenotypes (Chiasson et al., 1999; Laywell et al., 2000). In contrast, subependymal cells showed self-renewal properties (Chiasson et al., 1999; Morshead & van der Kooy., 2001) and multipotent characteristics (Chiasson et al., 1999), therefore demonstrating that subependymal and not ependymal cells fulfil the characteristics of true stem cells. The discrepancy between the reports could be a result of dissection protocol used by Johansson et al for the isolation of the ependymal cell layer. They injected Dil, a fluorescent lipophilic dye, into the lateral ventricle of rodents to label ependymal cells, which were then isolated. However, while ependymal cells were accurately labelled, it is possible that the dye also labelled the adjacent subependymal cells and resulted in the isolation of both cell types. The stem cell properties observed in their study could therefore reflect the presence of subependymal and not ependymal cells (Morshead & van der Kooy., 2001).

Interestingly, other studies have not only agreed with the finding of Chiasson et al, but also, it has been reported that the adult stem cell is a type of astrocyte, since glial fibrillary acidic protein (GFAP) immunopositive cells give rise to olfactory bulb neurones in vivo (Doetsch et al., 1999) and multipotent neurospheres in vitro (Doetsch et al., 1999; Laywell et al., 2000). Similarly, astrocytes of the dentate gyrus have also shown to generate neurones (Seri et al., 2001). From these results, it has been suggested that these neurogenic astrocytes may be derived from neurogenic radial glial cells, which exist at earlier developmental periods (Alvarez-Buylla et al., 2001). Regardless of cell identity and origin, the generation of neurones from adult neural progenitor cells has generated considerable excitement, not only because these cells offer another alternative source of neurones for cell replacement therapies, but also their use avoids many of the difficult ethical issues that are currently limiting the progression of embryonic and foetal stem cells. The use of adult neural progenitor cells also offers the possibility of autologous transplantation, thus avoiding the rejection issues associated with transplanting foreign tissue. However, as yet, no
evidence for the generation of neurones with dopaminergic phenotypes from adult neural stem cells has been reported. There are also limited grafting studies showing the behaviour of these cells in vivo. One study has showed that expanded adult neural progenitors from dentate gyrus differentiate into neurones only when injected into the dentate gyrus (Gage et al., 1995b), while another has shown that adult neural progenitor cells can behave like endogenous stem cells and migrate along the rostral migratory stream and differentiate into olfactory bulb neurones when grafted into the SVZ (Suhonen et al., 1996). More recently, grafts of adult neural progenitor cells into the intact or 6-OHDA lesioned striatum demonstrated good survival and migration but extremely low levels of neuronal differentiation (Dziewczapolski et al., 2003). Therefore, for adult neural stem cells to be a valid source of neurones for transplantation, research must focus on enhancing both the generation of neurones and differentiation of these cells into dopaminergic phenotypes. Once this is achieved, demonstrating the functionality of these neurones in animal models of PD is an essential prerequisite. Interestingly, the ability of endogenous adult neural stem cells to generate new neurones in response to ischemic insults (Arvidsson et al., 2002; Nakatomi et al., 2002), and infusions of certain growth factors (Kuhn et al., 1997; Fallon et al., 2000; Pencea et al., 2001), has resulted in the exciting possibility of simulating endogenous stem cells for the induction of self-repair. Adult neural stem cells therefore remain an attractive option. However, substantial progress in this field needs to be made before these cells can be considered as a practical option in the treatment for Parkinson's disease.

1.5.5 Plasticity of multipotent stem cells

Multipotent stem cells were originally thought to be restricted to forming only cell types of the tissue in which they reside. Recent experimental evidence however, has questioned this concept, with a plethora of reports showing that multipotent stem cells have broader lineage potentials, and respond to regional environmental cues to differentiate into a variety of other cell types.

This remarkable plasticity of stem cells has been demonstrated with cells isolated from a diverse range of tissues (for review see Verfaillie, 2002). For instance, bone marrow stem cells can differentiate into muscle cells and participate in muscle regeneration following injection into damaged muscles (Ferrari et al., 1998), mouse skeletal muscle cells can differentiate into a range of blood cells following injection
into the haematopoietic system of irradiated mice (Jackson et al., 1999) and haematopoietic stem cells can generate cells of the liver when intravenously injected into a mouse model of liver disease (Lagasse et al., 2000). Neural stem cells, which under normal circumstances give rise to neurones, astrocytes and oligodendrocytes, have also shown the ability to transdifferentiate and are capable of generating cell types from all three germ layers when injected into the developing chick embryo (Clarke et al., 2000). These stem cells also generate skeletal muscle both in vitro and when injected into regenerating tibialis anterior muscle of the adult mouse (Galli et al., 2000), and blood cells when injected into the haematopoietic system of irradiated mice (Bjornson et al., 1999). The generation of neural phenotypes from stem cells of non-neural origins has also been reported and has consequently opened up the possibility of using non-neural cells to provide a source of neurones for transplantation studies. Neural cells have been generated from isolated human umbilical cord blood cells, which have been shown to express markers associated with neurones and glial cells following exposure to specific culture environments containing a mixture of growth factors (Sanchez-Ramos et al., 2001; Bużańska et al., 2002). Bone marrow stem cells also express markers for neural precursors, neurones and astrocytes when expanded in epidermal growth factor (EGF), and subsequent differentiation in media containing brain derived neurotrophic factor (BDNF) (Sanchez-Ramos et al., 2000). Bone marrow stem cells grafted into the brain can adopt neural phenotypes, with intraventricular grafts of bone marrow cells into neonatal mice showing extensive migration and the generation of astrocytes (Kopen et al., 1999). Interestingly, human bone marrow stem cells grafted into the cortex of experimental rat stroke models generate functional neural cell types that improve the neurological deficits induced by cortical ischemia (Zhao et al., 2002). Functional recovery of motor and cognitive deficits is also observed when adult bone marrow cells are intravenously injected into mouse models of traumatic brain injury (Lu et al., 2001).

Collectively, these studies indicate that multipotent stem cells are extremely responsive to the environment in which they are placed, and that cells destined to generate cells of a particular type could be influenced down alternative pathways, i.e. multipotent stem cells show extensive plasticity. The discovery that non-neuronal stem cells can transdifferentiate into functional neuronal phenotypes also provides an attractive source of neurones for cell replacement therapies, particularly because these
non-neuronal stem cells are relatively more accessible than neural stem cells, and they offer the possibility of autologous transplantation. However, while this cell plasticity has generated great excitement in the stem cell field, caution has also been raised, with the validity of transdifferentiation reports being questioned (Vogel, 2002; Wurmser & Gage, 2002). Since most reports identify cell types by either their morphological characteristics, or by the expression of particular proteins, it has been argued that cells may adopt some of the characteristics of other cells, rather than completing changing their phenotype, and therefore giving the impression of transdifferentiation (Anderson et al., 2001). It has been also suggested that plasticity of cultured stem cells could be a characteristic that is acquired through the in vitro proliferation of these cells, rather than being an original property of the stem cells (Andersen et al., 2001). Support for this cautious approach regarding transdifferentiation has been recently provided by examining the apparent pluripotency of adult bone marrow stem cells (Terada et al., 2002). In this study, adult bone marrow stem cells, tagged with the green fluorescent protein (GFP) marker, were cultured with embryonic stem cells and shown to generate a variety of different cell types including beating cardiac myocytes, thus indicating transdifferentiation. However, on closer inspection it was revealed that all transdifferentiated cells contained twice as many chromosomes than usual, implying that bone marrow and embryonic stem cells had fused together. The generation of the diverse range of cell types was therefore, probably a result of embryonic stem cell differentiation and not a consequence of adult bone marrow stem cell transdifferentiation. Cell fusion events have also been shown to be responsible for the generation of hepatocytes from bone marrow stem cells (Wang et al., 2003; Vassilopoulos et al., 2003), and the apparent pluripotency of mouse neural stem cells when cultured with embryonic stem cells (Ying et al., 2002).

Since the frequency at which cell fusion occurs is lower than the reported rate of transdifferentiation (Wurmser & Gage, 2002), the possibility of transdifferentiation events cannot be excluded, however, future transdifferentiation studies must proceed with caution. Identifying converted cells by merely examining protein expression and cell morphology is insufficient, and more rigorous criteria that include testing whether the converted cells are functional must be used to conclude that total conversion of cells has been achieved.
1.6 The aims of this thesis

The research outlined above clearly demonstrates that neurotransplantation can be successful in the treatment of Parkinson's disease under appropriate conditions, and that neural progenitor cells have the potential to provide a source of neurones for this therapeutic strategy. However, as yet, insufficient neuronal and dopaminergic differentiation has been achieved using these cells, and the survival of dopamine neurones following transplantation is poor. The following experiments have therefore attempted to address these issues by looking at the parameters which affect progenitor cell differentiation, and the effects of antioxidant treatment on dopamine survival. In chapter three, I assess the capacity of rat VM, WGE & CTX neural progenitor cells, isolated from a range of embryonic ages to differentiate into neurones following different periods of in vitro expansion. In chapter four, the effects of ascorbic acid on the survival of dopamine neurones is examined. Since the in vitro dopaminergic differentiation of adult neural progenitor cells has not been reported, chapter five evaluates whether these cells have the potential to do so. The following two chapters (chapters six & seven) focus on validating a mouse animal model of PD suitable for the efficient assessment of the functional potential of mouse embryonic stem cells. In chapter six, a unilateral 6-OHDA-lesion mouse model has been established, which shows appropriate motor asymmetry following amphetamine stimulation, and in chapter seven, the ability of this model to demonstrate graft functionality by attenuating rotational bias is assessed.

The results from these experiments will be of fundamental use in future research studies, and will also address key issues that need to be resolved before the use of stem cells will be of any value in clinical trials of Parkinson's disease.
Chapter Two

Materials and Methods

This chapter describes the various techniques used in this thesis. The dissections, *in vitro* and surgical techniques employed are discussed in detail. The recipes for all buffers, solutions etc., can be found in the appendix.

2.1 Subjects

All experiments have been undertaken in accordance with the UK Animals (Scientific Procedures) Act 1986 and subject to local ethical reviews. Only female Sprague-Dawley rats (Harlan, UK) and female 129P2/OLA Hsd mice (Harlan, UK) were used in experiments presented in this thesis. Rats were housed in maximum groups of 4, and mice in groups of 6 per cage. Animals experienced a regular 12 hour light:12 hour dark cycle and had free access to food and water. When necessary, animals were killed using schedule 1 procedures that have been approved by the Home Office, UK.

2.2 Embryonic tissue dissection

2.2.1 Isolation of the embryonic brain

Following terminal anaesthesia and decapitation of mother (schedule 1), pregnant rats/mice were laid on their back and the hair from the abdomen was trimmed, swabbed with betadine then 70% alcohol. The peritoneum was opened using a vertical midline cut first through the skin and then through the fascia of the abdomen. Using clean forceps each uterine horn was gently lifted and the tissue attaching the uterine horns to the abdomen was trimmed off using sterile scissors. Embryonic sacs were removed from the uterine horns and transferred to a 50ml tube containing fresh sterile Hanks buffered saline solution (HBSS, Invitrogen, Paisley, UK). In the dissection hood, embryonic sacs were transferred into a Petri dish where embryos were removed from their amniotic sacs and transferred into fresh sterile HBSS. At this point the embryonic age of the embryos was confirmed by measuring the Crown-Rump length (CRL) of each embryo (Fig. 2.1a). The CRL for embryos aged E11, E12, E14, E16,
E18 & E20 are 6, 9, 11, 15, 17 & 19mm respectively. Once the embryonic age was confirmed the brain was removed (Fig. 2.1a-c). For compliance with legislation, embryos older than E11 are killed by decapitation before brains were removed. The method for brain removal was dependant on embryonic age. For embryos under the age of E18, the embryo was placed with the head on the side, and a vertical cut was made with a scalpel. The scalpel was drawn along the base of the brain just over the eye and snout. Care was taken not to damage the VM or the base of the forebrain. In some cases, the brain emerges and is removed, if not, a pair of fine forceps was used to slide up under the skull at the start of the cut and the skin and meninges were gently peeled away, allowing easy removal of the brain (Fig. 2.1a-c). For older embryos/neonates (E18 onwards), after decapitation the thin layer of skull was removed by a midline cut from the spine to the snout, along the dorsal surface of the brain by using small scissors. The skin and skull were then peeled back each side to expose the brain. The brain was then carefully removed and transferred to a 50 ml tube containing sterile HBSS kept on ice. The brains were rinsed by transferring brains through two Petri dishes containing sterile HBSS.

2.2.2 Dissection of the Ventral mesencephalon (VM)
The isolated brains were laid on their side and two vertical cuts were made at each ends of the mesencephalic flexure. Two horizontal cuts were also made one-third up of the dorsal and ventral surface of the flexure, which ran along the length of the VM and between the two vertical cuts (Fig. 2.1d-f). The VM was then separated from its surrounding meningeal layer by using forceps to tease the two layers apart, and VM tissue was transferred to small sterile Eppendorfs containing HBSS, kept on ice.

2.2.3 Whole ganglionic eminence (WGE) and cortex (CTX) dissection
The brains were laid dorsal side up and the striatal primordial was exposed by making a longitudinal cut around the midline of the cortex. The cortex was then cut away. When removing the whole ganglionic eminence (WGE), a horizontal cut was made underneath the heart-shaped structure, with care taken to avoid going too deep into the underlying cortex. If removing the LGE or MGE selectively, first a vertical cut is made to divide the LGE from MGE then a horizontal cut is made to remove the tissue
Chapter Two

(Fig. 2.1h-j). The pieces were transferred to small sterile Eppendorfs containing HBSS, kept on ice.
Figure 2.1 Removal and dissection of the rodent embryonic brain. Embryonic age is confirmed by measuring the Crown-Rump length (CRL) of each embryo (a). The embryos are killed by decapitation (this is required by the Animals Scientific Procedures Act 1986 for embryos older than E11). A cut is made across the eye and snout (a), and forceps are used to remove the skin (b). The brain is fully exposed and is removed ready for dissection (c). To dissect the ventral mesencephalon (VM) (d-f), two ventral cuts at the ends of the mesencephalic flexure (1 & 2 in d), and two horizontal cuts are made, one on the dorsal surface (3 in d) and one on the ventral surface of the brain (4 in e). The butterfly shaped VM tissue is then removed (f). The lateral view (g) shows the angles of the two vertical cuts and the location of the substantia nigra (sn). To dissect the cortex and the striatal eminence a longitudinal cut is made along the cortex, close to the midline (h), this is gently opened up to reveal the striatal primordia, and the cortex is cut away (i). To remove the whole ganglionic eminence (WGE), a horizontal cut is made underneath the heart-shaped structure (j). (Figure adapted from Dunnett & Björklund 1992).
2.3 

2.3.1 Isolation of the adult brain and dissection of hippocampal tissue

Following schedule 1, 6-week-old Sprague Dawley rats (Harlan, UK) were decapitated and the skin on the head was vertically cut from the spine to the snout to expose the skull. Using rongeurs, the skull was peeled away with care taken to avoid damage the underlying brain. The brain was then carefully removed and transferred to a 50 ml centrifuge tube containing sterile HBSS (Invitrogen, Paisley, UK) kept on ice. Adult brains were bisected into separate hemispheres and each hippocampal lobe was separated from the overlying cortex using the natural separation line along the alveus hippocampus (Palmer et al., 1999). The hippocampi were placed in cold sterile HBSS and then diced into fine pieces using scalpel blades and placed into a 15ml centrifuge tube containing sterile HBSS.

2.4 In vitro techniques - Embryonic tissue

2.4.1 Embryonic Tissue dissociation

Tissue in the Eppendorfs was washed three times with sterile HBSS by using a P1000 Gilson pipette. Care was taken not to suck up the pieces into the pipette. The final wash was removed and tissue was enzymatically digested using a solution of 0.1% trypsin (Worthington, Lakewood, USA) and 0.05% DNase (Sigma), at 37°C for 20 minutes. The trypsin/DNase solution was removed and the dissected tissue was washed three times with 200µl 0.05% DNase (Sigma). The final wash was replaced with a 200µl volume of DNase (Sigma) and the tissue was mechanically triturated into single cells using a P200 Gilson pipette set at approximately 160µl. 10-15 (max) aspirations were done initially, and any pieces were left to settle. Further aspirations were done if pieces of tissues remained. To determine the number of cells within the suspension a cell count was necessary (See section 2.6.1).

2.4.2 Cell Plating, maintenance and differentiation of embryonic cells

Cells were plated out at an initial density of 100,000 cells per ml of growth media, and were seeded in a 25cm² flask. Thereafter, at each passage, new flasks were seeded with 50,000 cells per ml. Growth media consisted of neurobasal medium (Invitrogen), supplemented with 1% B27 (Gibco), 1% antibiotic/antimycotic solution (Invitrogen), 30mM glucose (Sigma), 1mM glutamine (Invitrogen), 5mg/ml heparin (Sigma) and 20ng/ml human recombinant bFGF (R&D Systems, Abingdon, UK).
The flasks were then placed in the incubator set at 37 °C, 5% CO₂, and 2ml of growth media were added to cell cultures every other day. When cells became confluent or when neurospheres grew to a pre-defined size, cells were passaged (see section 2.4.3) and re-plated at a density of 50,000 cells/ml. At the same time, 50,000 cells in 30μl differentiation media was plated into wells of a 24-well plate containing cover glass slips (13mm diameter) coated with 100μl/ml polylysine (Sigma) and 10μl/ml laminin (Sigma). Cells were left in wells for 4-6 hours before 0.5ml differentiation media was added, this allowed for sufficient cell attachment. Wells were supplemented with 0.5ml differentiation media every other day following seeding. Differentiation media consisted of neurobasal medium (Invitrogen), supplemented with 1% B27 (Gibco), 1% antibiotic/antimycotic solution (Invitrogen), 30mM glucose (Sigma), 1mM glutamine (Invitrogen), 50ng/ml bovine serum albumin (Sigma), and 1% foetal calf serum (Invitrogen).

2.4.3 Embryonic cell passaging

When cells become confluent, or neurospheres reached a pre-defined size (typically 0.8-1mm diameter), cells were transferred from their flasks to 50ml centrifuge tubes. Neurospheres and media were withdrawn from their flasks using a pipette, but those cells which had adhered to the surface of the flasks were scraped off by using cell scrapers, and then media was pipetted into a 50ml centrifuge tube. Cells were then centrifuged at 700 rpm for 6 minutes, resulting in cells forming a pellet. The supernatant from the centrifuged tubes were poured off into a waste bottle, with the final drops of supernatant removed by using a P200 Gilson pipette set at 100μl. Care was taken not to dislodge the pellet of cells. 100μl of HBSS was added to the pellet and it was gently dislodged. All cells were drawn up into the pipette tip and transferred into a sterile 1.5ml Eppendorf. Any further media and cells from the tube was removed and placed into the Eppendorf. A further 100μl of HBSS was used to wash down the side of the tube to get all cells, and again transferred into the Eppendorf. Using a P200 Gilson set at 160μl the cells were mechanically dissociated by ~25 aspirations of the pipette. For the first 10 aspirations, the pipette tip was kept pressed against the bottom of the Eppendorf, it was then pulled up slightly for the remainder of the aspirations. The aspirations were quite fast, but care was taken not to
2.5 In vitro techniques - Adult tissue

2.5.1 Adult tissue dissociation

Dissected tissue was enzymatically digested using a solution of 0.01% Papain (Worthington Biochemicals, Freehold, NJ). 0.1% neutral protease (Dispase; Boehringer Mannheim, Indianapolis, IN) and 0.01% DNase (Worthington Biochemicals, NJ) dissolved in HBSS, at 37°C for 30-45 minutes. The PPD solution was removed and replaced by 5-10ml DMEM/F12 (1:1, Invitrogen, Paisley, UK) containing 10% FBS (Invitrogen, Paisley, UK). Tissue was mechanically dissociated into single cells by gentle trituration using a 10ml pipette. A maximum of 10-15 aspirations were done before tissue was centrifuged at 400 x g for 5 minutes. The supernatant was removed and the trituration, washing and centrifugation steps were repeated at least two more times to ensure the complete removal of PPD and maximum tissue dissociation. After the final wash, the supernatant was removed and tissue was fractionised using the Percoll buoyancy fraction method (Palmer et al., 1999). Tissue was mixed with 35% isotonic Percoll solution (Amersham Pharmacia Biotech, Uppsala, Sweden) and the cell suspension was then fractionated by centrifugation for 10 minutes at 1000 x g. 100% isotonic Percoll solution was made by mixing nine parts of Percoll with one part of 10 x PBS and was diluted to the desired concentration by the addition of DMEM/F12 containing 10% FBS. Floating myelin and tissue debris were discarded and the cell pellet resuspended in 65% Percoll solution and fractionated again by centrifugation for 10 minutes at 1000 x g. Any remaining floating debris were removed and the floating neural progenitors (~2ml above pellet of red blood cells) were collected and washed free of Percoll by centrifugation in DMEM/F12 containing 10% FBS at 400 x g for 5 minutes in 15ml centrifuge tubes. The supernatant was removed and cells resuspended in 200µl DMEM/F12 containing 10% FBS. To determine the number of cells a cell count was required (see section 2.6.1).
2.5.2 Cell Plating, maintenance and differentiation

Adult progenitor cell suspension was plated on poly-L-ornithine (10\(\mu\)g/ml, Sigma) and laminin (5\(\mu\)g/ml, Sigma) coated 100mm Petri dishes at a density of 500,000 cells per dish in DMEM/F12 containing 10% FBS. The dishes were placed in the incubator set at 37\(^\circ\)C, 5% CO\(_2\). After 24 hours, media is removed and replaced by growth media consisting of DMEM/F12 supplemented with 1% N2 (Invitrogen, UK) and 20ng/ml bFGF (R&D Systems, Abingdon, UK). Most cells will be non-adherent so care must be taken when removing media. 75% growth media is replaced every other day, with removed media kept as ‘conditioned media’. When cells became confluent the cells were passaged see section 2.5.3 and re-plated at 250,000 cells/dish. At the same time, 25,000 cells in 30\(\mu\)l differentiation media were plated into Falcon 8-chamber culture slides, coated with poly-L-ornithine (10\(\mu\)g/ml) and laminin (5\(\mu\)g/ml). Cells were left in chambers for 4-6 hours before 0.5 ml differentiation media was added, this allowed for sufficient cell attachment. Chambers were supplemented with 0.5ml differentiation media every other day following seeding. Differentiation media consisted of DMEM/F12 supplemented with 1% N2, 1% FCS and 0.5\(\mu\)g/ml trans- retinoic acid (Sigma).

2.5.3 Cell passaging

When the adult cells become confluent, media were removed and the dish was rinsed with 0.025% trypsin solution (Worthington, Lakewood, USA). Trypsin was immediately removed and dish incubated at 37\(^\circ\)C for 1-2 minutes until the cells began to detach. Cells were flooded with conditioned medium and the dish gently tapped to loosen cells. The conditioned media and cells were transferred to a 15ml centrifuge tube, and centrifuged at 400x\(g\) for 5 minutes to pellet cells. The supernatant from the centrifuged tubes was poured off into a waste bottle, with the final drops of supernatant removed by using a P200 Gilson pipette set at 100\(\mu\)l. Care was taken not to dislodge the pellet of cells. 100\(\mu\)l of conditioned media was added to the pellet and it was gently dislodged. All cells were drawn up into the pipette tip and transferred into a sterile 1.5ml Eppendorf. A further 100\(\mu\)l of conditioned was used to wash down the side of the tube to get all cells, and again transferred into the Eppendorf. Using a P200 Gilson set at 160\(\mu\)l the cells were mechanically dissociated by ~10 aspirations of the pipette. The aspirations were quite fast, but care was taken not to get too many air
bubbles in the medium. A cell count was then taken (see section 2.6.1) and cells were resuspended in fresh growth media supplemented with 20% conditioned media and re-plated at a density of 25,000 cells/dish.

2.6 General In vitro techniques

2.6.1 Cell counting

Counting the number of cells in the suspension was achieved by using a haemocytometer. Because of the substantial numbers of cells in the suspension, the cell suspension was diluted prior to the cells being counted. A dilution factor of 50 was often used and was achieved by the following method:

10μl cell suspension was transferred into 40μl HBSS and mixed thoroughly. 10μl solution was taken from this cell/HBSS mixture and put into another 40μl of fresh HBSS. 10μl was then taken from this mixture and put into 10μl trypan blue by using a P20 Gilson set at 10μl. With the Gilson set at 10μl, the cells were mixed with the HBSS and trypan blue three times. 10μl of cell/trypan blue solution was drawn up and transferred under the cover slip of the haemocytometer and cells were viewed using an Olympus stereo microscope.

The grid on the haemocytometer has 25 large squares sub-divided into 16 smaller squares. The whole grid was counted. When counting the cells the inclusion/exclusion rule was applied – thus cells touching any of the three lines of the upper edge and left hand side where counted, but cells that touched any of the three lines on the right hand and lower edge were not, even if they were inside the grid. If clumps of cells were observed under the microscope, the original suspension was dissociated further by more trituration and the counting procedure was repeated. Both live and dead cells were counted (live cells are white whereas dead cells stain blue due to the uptake of trypan blue through damaged cell membranes), and the total number of viable cells present in one μl of the cell suspension was calculated using the following formula:

\[
\text{No. live cells/μl} = \frac{\text{number of white cells in whole grid} \times \text{dilution factor}}{10^2}
\]

2.6.2 Immunocytochemistry and microscopy

After 7 days differentiation, each well containing cells in the 24-well plate or chamber slides was emptied of its media and was washed once with either 0.1M phosphate buffered saline (PBS) or HBSS solution (see appendix for further details of solutions).
PBS or HBSS was removed and cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at 4°C (for GABA staining, cells were fixed a solution of 4% paraformaldehyde and 1% glutaraldehyde for 20 minutes at 37°C). PFA was removed and cells washed three times with PBS. Cells were then permeabilised with 100% ethanol for two minutes. Following 3 washes in PBS, coverslips/chambers were pre-incubated in 5% normal goat serum (NGS, Dako, Denmark) in PBS for 1 hour at 4°C. Coverslips or chambers were incubated with a range of antibodies (β-tubulin-III, 1:400, Sigma; TH, 1:1000, Chemicon; GABA, 1:100, Diasorin Inc., Stillwater, USA; GFAP, 1:2000, Dako, Denmark) in 1% NGS in PBS and left over night at 4°C. Primary antibody was not added to at least one well of each condition, this acted as the control. Following 3 washes with PBS, coverslips/chambers were incubated with Alexa 488 goat anti-mouse secondary antibody (1:200, Molecular probes, USA) & Alexa goat anti-rabbit secondary antibody (1:200, Molecular probes, USA) with 1% NGS in PBS or 2 hours at 4°C in the dark. Coverslips and chambers were rinsed with PBS then treated with 1.25μl/ml of 0.2mg/ml Hoechst (Fisher, Acros Organics, Loughborough, UK) for 4.5 minutes. Following 3 final washes in PBS, coverslips were mounted onto uncoated glass microscope slides and chamber slides were covered with coverglass using PVA-DABCO mountant and stored at 4°C.

Cells were visualised under UV fluorescence using a Leitz stereomicroscope. Initially UV was used to identify Hoechst stained nuclei to obtain a total cell count within a grid randomly placed on the coverslips and viewed under 50x magnification. Each cell immunopositive for Hoescht was checked for expression of β-tubulin under fluorescence. Cells immunopositive for β-tubulin were also checked for expression of TH or GABA under a different UV wavelength. Counts were made on at least 5 fields per coverslip, including a minimum of 250 cells, and on replicates of 3-4 coverslips per treatment.

2.7 Surgical procedures

2.7.1 6-Hydroxydopamine (6-OHDA) lesion

Female Sprague-Dawley rats (~250g) and female 129P2/OLA Hsd mice (~25-30g), were anaesthetised using gaseous isoflurane (2-5% in 2:1 O₂:N₂) and received unilateral stereotaxic injections of 6-hydroxydopamine hydrobromide (6-OHDA, Sigma). Rats received lesions into the right medial forebrain bundle (anterior -4.4,
lateral -1.8, ventral -7.8, incisor bar -2.3), with mice receiving lesions in either the right medial forebrain bundle (anterior -2.0, lateral -0.7, ventral, -4.8, incisor bar 0.0) or the right midstriatum (anterior 0.4, lateral -1.8, ventral -3.5, incisor bar 0.0).

Each animal received 4μg/μl of 6-OHDA dissolved in physiological saline containing 0.01% ascorbic acid. 6-OHDA was infused over a three-minute period for rats and over a one-minute period for mice, at a rate of 1μl/min. To allow sufficient diffusion of 6-OHDA, the cannula was left in place for at least 2 minutes before being slowly removed.

After lesioning, rats and mice were sutured and injected with 5ml or 0.5ml 0.9% saline/glucose solution respectively to prevent dehydration. The drinking water was supplemented with Paracetamol for the following 48 hours and animals were carefully monitored post-surgery. All animals were allowed to recover for at least 10 days before behavioural testing commenced.

2.7.2 VM cell grafts in 6-OHDA model

Before cell grafting, VM tissue was dissected from E14 rat or E13 mouse embryos and a single cell suspension was prepared as described previously (section 2.2.2). Lesioned animals were anaesthetised using gaseous isoflurane (2-5% in 2:1 O₂:N₂), and VM cells were grafted into the right midstriatum (rats: anterior 0.8, lateral -3, ventral -5 & -4.5, incisor bar -2.3; mice: anterior 0.8, lateral -1.8, ventral -3 & -2.5, incisor bar -0.0). The cell suspension contained 125,000 cells/μl, with a total of 500,000 being deposited (2μl at each depth) using a Hamilton microsyringe over a 4 minute period. The syringe was left in place for a further 4 minutes to allow for the diffusion of the suspension and to prevent withdrawal of suspension along the needle tract. After surgery, rats and mice were sutured and injected with 5ml or 0.5ml 0.9% saline/glucose solution respectively to prevent dehydration. The drinking water was supplemented with Paracetamol for the following 48 hours and animals were carefully monitored post-surgery. All animals were allowed to recover for at least 10 days before behavioural testing commenced.

2.8 Rotations

The induction of rotational bias in unilateral dopamine depleted animals following pharmacological stimulation was first described by the Swedish scientist Urban
Ungerstedt in the early 1970's (Ungerstedt et al., 1970; Ungerstedt, 1971a; Ungerstedt, 1971b). Since rotation reflects the amount of dopamine loss, this model provides a useful way of assessing the potential of curative treatments by examining the attenuation of rotational behaviour. This model has therefore been extensively used throughout this thesis to assess the potential of stem cells, progenitors and neurones to provide functional recovery and thus alleviation of rotational bias.

2.8.1 Behavioural testing: Rats

2.8.1.1 Amphetamine and Apomorphine-induced rotations

Amphetamine and apomorphine-induced rotational behaviour was assessed only after full recovery from 6-OHDA lesion surgery. Rats were placed in automated rotation bowls with a diameter of 32cm and height of 14cm and allowed to habituate for 10 minutes before being injected intraperitoneally with either 2.5mg/kg of methamphetamine (Sigma) or subcutaneously with 0.05mg/kg (dissolved in 0.2% ascorbic saline) apomorphine hydrochloride (Sigma). Rats were monitored for either 90 minutes (amphetamine-induced rotations) or 60 minutes (apomorphine-induced rotations) in a closed room to avoid any environmental disturbance. The difference in rotation times was due to the difference in the metabolism of the two drugs. At any one time, 8 rats were rotated simultaneously and the extent of dopamine depletion was calculated as a measure of total full body turns. Both ipsilateral and contralateral rotations were recorded automatically by an on-line connection to a computer (Rotometer activity system, Cambridge, UK). At the end of the testing interval, net turns in the dominant direction were calculated.

2.8.2 Behavioural testing: Mice

2.8.2.1 Amphetamine and Apomorphine-induced rotations

Amphetamine and apomorphine-induced rotational behaviour was assessed only after full recovery from 6-OHDA lesion surgery. Mice were placed in cylinders with a diameter of 11.5cm and height of 14cm and allowed to habituate for 10 minutes before being injected with either a dose of d-amphetamine (Sigma) i.p or with apomorphine hydrochloride (Sigma) s.c.
Mice were monitored for either 90 minutes (amphetamine-induced rotations) or 48 minutes (apomorphine-induced rotations) in a closed room to avoid any environmental disturbance. The difference in rotation times was due to the difference in the metabolism of the two drugs. At any one time, 6 mice were rotated simultaneously and the extent of dopamine depletion was calculated as a measure of total full body turns. Both ipsilateral and contralateral rotations were recorded over the testing interval, with net turns per minute calculated in the dominant direction. Each mouse was observed for 1 minute every 6th in a cycle order and rotations were recorded. Therefore a total of 15 x 1 minute recordings were made for each mouse during amphetamine-induced rotation and 8 x 1 minute recordings during apomorphine-induced rotation. Rotation values for each mouse were totalled at the end of each run, and values averaged to give the average rotation per minute.

2.9 Histological procedures

2.9.1 Perfusion and sectioning of 6-OHDA lesioned brains

After behavioural testing, animals were terminally anaesthetised with 0.5ml Euthatal and transcardially perfused with phosphate buffered saline (PBS), pH 7.4 for 1 minute, followed by fixation in 4% paraformaldehyde (PFA), pH 7.4 for 2 minutes for mice or 3-4 minutes for rats. The brains were removed and post fixed in PFA for a further 24 hours before being placed in PBS containing 25% sucrose. The brains were left overnight at room temperature by which time the brains had sunk and were ready for sectioning.

Brains were coronally sectioned on a microtome at 40μm and stored in 96 well plates at 4°C in Tris buffered 0.9% saline (TBS), pH 7.4, containing 0.5% sodium azide. Sodium azide was added to inhibit bacterial and fungal growth that may damage the tissue sections. While sectioning the midbrains, the non-lesioned side (left side) was marked by pushing a fine needle through the brain towards the cerebellum, and a cut was made on the left hand side cortex. This provided a clear marking which distinguished the lesioned side from the non-lesion side of the brain.

2.9.2 Immunohistochemical staining of brain sections

Selected brain sections were quenched with 10% hydrogen peroxide and 10% methanol in distilled water for 5 minutes followed by three 10-minute washes in Tris
Buffered Saline (TBS). Sections were then placed in TXTBS (TBS containing 0.2% Triton X-100) containing 3% normal goat serum (NGS) for 60 minutes. Without washing, brain sections were incubated overnight at room temperature, or over 2 nights at 4°C in TXTBS containing the primary rabbit anti-tyrosine Hydroxylase (TH) antibody at a concentration of 1:1000 (Chemicon int., Temecula, CA). After three 10 minute washes with TBS, sections were incubated at room temperature for 3 hours in the secondary biotinylated goat anti-rabbit antibody at a concentration of 1:200 (DAKO, Denmark) in TBS. Sections were washed in TBS before being incubated for a further 2 hours at room temperature in strepavidin-biotin complex (DAKO) in TBS with 1% normal goat serum. After three 10 minutes washes in TBS, sections were washed in TNS (distilled water containing 0.6% trizma base), and left overnight at 4°C. TH positive cells in the substantia nigra were visualised by the reaction with vector SG kit (DAKO) until appropriate colour change had taken place. Washing sections twice in TNS stopped the reaction.

Sections were mounted on gelatinised glass slides, air dried overnight and dehydrated in an ascending series of alcohols, cleared in xylene, and coverslipped with DPX.

2.9.3 Lesion analysis

Counting the number of TH positive cells in the substantia nigra was achieved by counting every third 40μm coronal brain section at a magnification of 250X using a Leitz Dialux 22 microscope. Only those cells that were clearly stained and with dendritic processes were counted on both the lesioned and non-lesioned side of the brain. Counting cells on the non-lesioned side served as a control. Segregation of the ventral tegmental area (VTA) and the substantia nigra was achieved by using the medial terminal nucleus of the accessory optic tract as a landmark, as described by Lee et al., (1996) and Bensadoun et al., (2000).

TH cell numbers on the lesioned and non-lesioned sides of the brain were totalled and the percentage TH loss on the lesioned side was calculated, thus giving an indication of the degree of lesioning. Figure 2.4 shows photographs of a typical 6-OHDA lesioned mouse brain. There is dopamine depletion at the striatal level, and obvious dopamine reduction in the substantia nigra on the lesioned side. The medial terminal nucleus of the accessory optic tract, which separated the VTA from substantia nigra, is also shown.
Figure 2.4 Unilateral 6-OHDA lesions of the 129 OLA mouse brain. Unilateral lesions of dopaminergic neurones are clearly visible due to the reduction of tyrosine hydroxylase staining on the lesioned side (right). At both the levels of the striatum (A) and the substantia nigra (SN) (B), the lesioned side is depleted of dopamine. Cutting the cortex and punching the brain on the non-lesioned side provided a suitable marking confirming the lesioned and non-lesioned side (black arrows in B). The medial terminal nucleus of the accessory optic tract (MTN) was used as a landmark allowing the distinction between the dopamine neurones of the ventral tegmental area (VTA) and the substantia nigra (C). Only cells with clearly labelled cell bodies and dendritic processes where counted (D). Scale bars in A & B = 1mm, C = 0.5mm, D = 100μm.
2.9.4 Striatal dopaminergic graft analysis

Counting the number of TH positive cells in the striatum was achieved by counting every sixth 40μm coronal brain section at a magnification of 200X using a Leitz Dialux 22 microscope. Only those TH cells that were clearly stained and with dendritic processes were counted. Once sections from a single animal brain were counted, the number of TH cells from each section was combined, thus giving the total number of TH cells per animal. The average TH cell diameter was then calculated by measuring at least 50 random TH positive cells using an Olympus BX50 microscope and an Olympus C.A.S.T grid system.

The total number of TH cells present in each brain was calculated using the Abercrombie correction formula (Abercrombie., 1946).

Abercrombie correction formula:

\[
\text{Total number of cells} = F \times \frac{A \times M}{(D+M)}
\]

Where
- \(F\) = Frequency of sections i.e. 1 in 6
- \(A\) = Cell counts for entire animal
- \(M\) = Section thickness
- \(D\) = Average cell diameter

Figure 2.5 shows photographs of a typical grafted rat brain. Grafts of dopamine neurones in the denervated striatum are shown and individual TH positive dopamine neurones are observed at higher magnification.

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Figure 2.5 VM grafts into the striatum of the rodent brain. Figure A shows the striata of a non-lesioned brain. TH expression in both the left and right striatum is evident (A). Unilateral 6-OHDA lesions of the medial forebrain bundle results in decreased TH expression in the striatum as shown in B. Figure B also shows the presence of grafts of VM tissue within the striatum and the characteristic halo of TH innervation. At higher magnification, the graft is more evident (C), and individual TH neurones derived from the grafts are clearly visible (D). Scale bars in A & B = 1mm, C = 0.5mm, D = 100µm.
Chapter Three

Experiment One - The effects of region, progenitor age and in vitro proliferation on the neurogenic potential of rat neural progenitor cells

Summary

This chapter discusses the effects of progenitor age and prolonged in vitro proliferation on the neurogenic potential of neural progenitor cells. Neural progenitor cells were isolated from the developing rat VM, WGE and CTX, at ages ranging from E11 to E20, and cultured in vitro for up to 40 days. Following periods of proliferation, progenitor cells were differentiated into neural phenotypes, and the neurogenic potential of the progenitors was assessed by counting the number of cells immunopositive for the neuronal marker β-tubulin-III. The results from this study show regional differences in the neurogenic potential of progenitors, with cells from the WGE generally generating the highest percentage of neurones and progenitors from the VM generating the least. The yield of primary neurones and percentage neurogenesis of progenitor cells was also shown to differ with age, with the neurogenic capacity of WGE and CTX progenitors behaving similar to each other, but different to progenitor cells of the VM across the age range. A significant decrease in neurogenesis was also observed with all progenitors, at all ages after increasing time of expansion in vitro, with passage 5 progenitor cells generating ~2% neurones, compared to passage 1 progenitors where a maximum yield of 25% neurones was observed. From this assessment we propose that neural progenitor cells isolated from the same region but different ages, behave differently, and that the capacity of neural progenitor cells to differentiate into neurones is significantly reduced with increased time in vitro.
Chapter Three

3.1 Introduction

The initial reports showing the successful in vitro proliferation and neural differentiation of progenitor cells isolated from the embryonic and adult mouse brain (Murphy et al., 1990; Reynolds et al., 1992; Reynolds & Weiss, 1992) have provided a useful in vitro model for studying neural development.

With the establishment of this in vitro model, a plethora of reports have been published evaluating the characteristics of progenitor cells isolated from distinct areas of the rodent (Rosser et al., 1997; Tang et al., 2002; Smith et al., 2003; Dziewczapolski et al., 2003), pig (Armstrong et al., 2003) and human brain (Storch et al., 2001; Riaz et al., 2002; Wu et al., 2002; Li et al., 2005).

During proliferation, these progenitor cells typically form cell clusters or free-floating aggregates termed “neurospheres” (Reynolds & Weiss, 1992), with mitogens such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) being essential for the survival and proliferation of these cells (Murphy et al., 1990; Mytilineou et al., 1992; Vescovi et al., 1993; Qian et al., 1997). Subsequent differentiation into a host of neural phenotypes is achieved by mitogen withdrawal, although, neuronal differentiation down particular phenotypic lineages has been shown to be influenced by a variety of additional factors. This plasticity of progenitor cells has resulted in the manipulation of the culture conditions for optimal generation of specific neuronal phenotypes, in particular dopamine neurones that can be used to replace the dopaminergic neurones lost in Parkinson’s disease (Ling et al., 1998; Studer et al., 2000; Yan et al., 2001).

Neural progenitor cells have been shown to generate neurones in culture and when transplanted into the brain, therefore the use of progenitor cells as an alternative source of tissue for transplantation in neurodegenerative disease has great potential, providing that these progenitors can survive, integrate and generate functional dopamine neurones following grafting into the adult brain.

When expanded human and rat EGF-responsive progenitor cells, isolated from the developing mesencephalon or striatum, are transplanted into ibotenic acid or 6-OHDA lesioned rats however, there is poor survival of cells, resulting in thin grafts, limited migration into the surrounding host tissue and no attenuation of rotational behaviour (Svendsen et al., 1996). Similarly, grafts of expanded progenitor cells isolated from the developing pig ventral mesencephalon have also failed to show any
attenuation of rotational bias in 6-OHDA lesioned rats (Armstrong et al., 2003), suggesting that progenitor cells may not be capable of generating neurones that are functional. However, bFGF and EGF-responsive human progenitor cells, when expanded and grafted into the dopamine-depleted rats have shown more encouraging results. Not only were large grafts formed, but progenitor cells also showed migration and differentiation into astrocytes and functional dopamine neurones that reduced rotational bias (Svendsen et al., 1997a). Although only 2 rats showed a reduction in rotation, this report illustrates that expanded progenitor cells do have the ability to generate neurones capable of alleviating behavioural deficits. More recently, the differentiation of expanded rat VM progenitors into functional dopamine neurones has also been shown following transplantation into the brain, with resultant grafts being similar in size as those typically seen with grafts of primary tissue (Studer et al., 1998). In this study, progenitor cells were expanded and transplanted as differentiated floating aggregates, thus avoiding the enzymatic or mechanical dissociation of cells that can lead to destruction of axodendritic trees and cell death. Together these reports indicate that progenitor cells have the capacity to generate functional neurones, and therefore the use of progenitor cells in neurotransplantation strategies is a real possibility.

The potential of neural progenitor cells to generate neurones is apparent at a variety of ages, with progenitors isolated from E12 (Studer et al., 2000; Yan et al., 2001), E14 (Brundin et al., 1988; Ostenfeld et al., 1999; 2002), E16 (Brundin et al., 1988; Svendsen et al., 1997b), E18 (Svendsen et al., 1995), E20 (Brundin et al., 1988) and from the adult brain (Gage et al., 1995b; Palmer et al., 1995; 1997) all showing proliferation and differentiation into a range of neural phenotypes.

At present a clear difference in the neurogenic potential between embryonic and adult progenitors is apparent, with embryonic progenitors being able to generate abundant neurones, but adult progenitors showing restricted neurogenesis. However, it is currently unclear whether progenitor cells from the same region, but at different gestational ages are the same and have similar neurogenic potentials, or are different and have distinct properties. Previous reports indicate that age has a significant effect on the behaviour of progenitor cells, with progenitors isolated from the developing pig VM at ages corresponding to E11/12 & E14 in the rat, having different in vitro and in vivo characteristics (Armstrong et al., 2003). Primary rat VM-derived neurones
of different ages have also shown different properties, with neurones isolated at E14-16 showing functional recovery of behavioural deficits induced by 6-OHDA lesions, compared to neurones isolated at E20 that do not (Brundin et al., 1988). More recently, a report has shown the importance of the age of rat neural progenitor cells isolated from the VM at ages E12-E14. In this study, the size of neurospheres, percentage of neurones and TH positive neurones, as well as percentage of oligodendrocytes and astrocytes generated were dependent on progenitor age (O'Keeffe & Sullivan., 2004). Together these reports suggest that progenitors from different ages behave differently.

There is also a degree of uncertainty about the neurogenic potential of progenitor cells after prolonged periods in vitro (Smith et al., 2003). In theory, one of the characteristics of progenitor cells, which make them so appealing, is their ability to generate a continuous and plentiful source of neurones. If progenitor cells cannot fulfil this requirement, these cells will be of no benefit in providing an alternative source of neurones for transplantation.

To address these issues, progenitor cells have been isolated from the ventral mesencephalon (VM), whole ganglionic eminence (WGE) and the cortex (CTX) of the developing rat brain, at ages ranging from embryonic day 11 (E11) to embryonic day 20 (E20). Progenitor cells underwent 7 days differentiation at the time of dissection (primary cells), and following 7-10 days (passage 1), 21-25 days (passage 3) and 35-40 days (passage 5) of in vitro proliferation. The extent of neurogenesis has been assessed for progenitors at each age, region and passage, thus providing a large, parametric study on the neurogenic potential of rat neural progenitor cells.
3.2 Experimental Procedure

A detailed description of the dissection procedure and the in vitro techniques used in this chapter can be found in chapter two, section 2.2, 2.4 & 2.6.

Before dissection of the relevant brain tissue, the gestational age of the rat embryos was confirmed by measuring the crown-rump length (CRL, see chapter two, section 2.2). Dissected tissue was dissociated (chapter two, section 2.4) and proliferated in growth media consisting of neurobasal medium, supplemented with, 20ng/ml bFGF, 1% B27 (Gibco), 1% antibiotic/antimycotic solution (Invitrogen), 30mM glucose (Sigma), 1mM glutamine (Invitrogen) and 5mg/ml heparin (Sigma) at 37°C & 5% CO₂. Cells were carefully monitored in vitro, and when neurospheres reached a pre-defined size (typically 0.8-1mm diameter), the neurospheres were dissociated into single cells (passaged, see chapter two, section 2.5.3) by mechanical trituration and re-plated at 500,000 cells/ml, thus preventing death of those cells within the centre of the spheres. Cells were differentiated over a 7 day period in differentiation media consisting of neurobasal medium, supplemented with 1% B27, 1% antibiotic/antimycotic solution, 30mM glucose, 1mM glutamine, 50ng/ml bovine serum albumin, and 1% foetal calf serum. Primary cells were differentiated at the time of dissection (passage 0), and progenitors following 7-10 days (passage 1), 21-25 days (passage 3) and 35-40 (passage 5) days of proliferation. Following 7 days differentiation, cells were fixed in 4% PFA and stained with the marker β-tubulin-III for the identification of neurones. Neurones were also stained for TH and GABA expression (see chapter two, section 2.6.2).

To normalise the variance data was plotted on a log scale rather than a linear scale.

All experiments were repeated three times and statistical tests of analysis of variance (ANOVA) were analysed using the statistical package Genstat v7.2, with further analysis of the results being achieved by using the Neuman Keuls t-test.
Chapter Three

3.3 Results

3.3.1 Cell culture

Cells isolated from the ventral mesencephalon (VM), whole ganglionic eminence (WGE) and the cortex (CTX), at ages ranging from E11-20 all generated free-floating aggregates (neurospheres) within two days of *in vitro* proliferation (Fig. 3.1A). Neurospheres proliferated extensively *in vitro* and were dissociated into single cells (passaged) when they reached a pre-defined size, a point that was typically reached after approximately 7-10 days *in vitro*. Interestingly, cells isolated at ages ranging from E11-E16 generally proliferated faster than cells isolated at older donor ages and were subsequently passaged at earlier time points.

During differentiation, all cells formed monolayer cultures that adhered to the culture flasks, and based on their morphological characteristics, cultures of cells from all regions and ages contained different cell phenotypes. All neuronal phenotypes were identified using the neuronal marker β-tubulin-III (Fig. 3.1B). Dopaminergic and GABAergic neurones were also identified by examining neurones immunopositive for the antibodies tyrosine hydroxylase (Fig. 3.1C) and GABA (Fig. 3.1D) respectively.

Figure 3.1 Cell cultures of neural progenitor cells. All cells types proliferated as neurospheres (A). Following differentiation, neurones were identified using the neuronal marker β-tubulin-III and visualised using green fluorescence (B). Dopaminergic and GABAergic neurones were identified using TH (C) and GABA (D) respectively, and were visualised using red fluorescence. Scale bar = 0.5mm in A & 100μm in B, C &D.
3.3.2 Neuronal expression of primary cells

The generation of neurones from primary cells (cells differentiated at the time of dissection), isolated from the ventral mesencephalon (VM), whole ganglionic eminence (WGE) and cortex (CTX), at ages ranging from E11-E20 can be seen in Fig. 3.2. Since these cells did not undergo any in vitro expansion, the majority of the cells immunopositive for β-tubulin-III probably reflect the number of neurones isolated at the time of dissection, rather than the number being derived by neural progenitor cells. For this reason, primary cell data and data from expanded cells cannot be directly compared.

Primary cell cultures from all three regions, and at all ages showed large neuronal yields, with nearly all cultures yielding over 50% neurones, and some exceeding a neuronal yield of 70% (Fig. 3.2a). A significant difference in the percentage of neurones isolated from different regions was observed ($F_{(2,226)}=58.64$, $p<0.001$), with primary cells from the WGE and CTX showing higher neuronal yields compared to the cells from VM at most ages. A significant effect of embryonic age on neuronal yield was also seen ($F_{(6,226)}=12.52$, $p<0.001$). Peak neuronal expression of VM primary cells occurred at E14, with a decline in neuronal expression post E14 (Fig. 3.2a & 3.2b). At E20, primary VM cells showed significantly less neurones compared to the cells isolated from the VM at the ages E13 (NK: $t_{(6,6)}=6.391$, $p<0.05$) & E14 (NK: $t_{(6,7)}=8.355$, $p<0.01$) (Fig. 3.2a & b).

Neurones from WGE primary tissue at ages E11-13 showed little variation in neuronal numbers with all three ages showing approximately 60% neurones. The optimal age to isolate neurones from the WGE was at E14-18. At these ages, WGE primary cells yielded significantly more neurones compared to all other ages ($p<0.01$). At E20, primary cells expressed the same number of neurones as seen at E11-13 (Fig. 3.2a & c).

Primary cells isolated from the cortex (CTX) behaved in a similar fashion to primary WGE cells with significantly higher number of neurones being observed at ages E14-18 compared to all other ages ($p<0.05$). At earlier ages (E11-13), there were slight, but insignificant fluctuations in the percentage of neurones, with all three ages generating between 55-62% neurones. E20 primary cells showed similar properties to these early age cells by yielding approximately 57% neurones (Fig. 3.2a & d).
Figure 3.2 Neurogenic properties of primary cells isolated from three different brain regions (VM; black bars, WGE; grey bars & CTX; white bars) at different embryonic ages (a). Both a significant difference in neurogenic potential with region (p<0.001) and with embryonic age (p<0.001) was observed. Individual graphs showing the neurogenic properties of primary cells isolated from the VM (b), WGE (c) & CTX (d) at different embryonic ages are also shown. VM cells generate higher numbers of neurones at ages E14, with a reduction post E14 (b). Peak neurogenesis for primary WGE & CTX cells occurs at ages E14-18 (c & d), with cells at E20 showing a reduction in neuronal yields. Data expressed as mean ± S.E.M. across three separate experiments. **p<0.01 vs. E11, E12, E13 & E20, *p<0.05 vs. E11, E12, E13 & E20, **a p<0.05 vs. E13, **b p<0.05 vs. E14.
3.3.3 Generation of TH positive and GABAergic neurones from primary cells

The proportion of primary cell-derived neurones, which were of GABAergic and possible dopaminergic phenotypes, was determined by examining the number of neurones immunopositive for gamma-amino butyric acid (GABA) and tyrosine hydroxylase (TH) respectively (It is important to note that TH is not a specific dopaminergic marker and therefore all cells that are immunopositive for this antibody are only potentially of dopaminergic phenotypes) (Fig. 3.1C & D).

TH immunopositive neurones were only expressed in neurones derived from VM cells, and not in WGE or CTX primary neurones (Fig. 3.3a), whereas, GABA expression was evident in neurones derived from all three regions (Fig. 3.3b).

The percentage of TH positive neurones expressed by primary VM cells varied significantly with age ($F_{(6,98)}=33.09, p<0.001$), with values ranging from ~4-12% (Fig. 3.3a). Neurones derived from E12-14, generated significantly higher percentages of neurones immunopositive for TH compared to all other ages ($p<0.01$), with no significant difference between these ages being observed. At ages, E11, E16, E18 & E20, the percentage of TH positive neurones varied between 4-7% and were not significantly different from each other.

For the expression of GABA, only four age points (E12, E14, E16 & E20) for each region were compared. The percentage of neurones immunopositive for GABA ranged from ~1-17% (Fig. 3.3b), with a significant difference in GABA expression across the age range ($F_{(3,80)}=41.49, p<0.001$), region ($F_{(2,80)}=109.56, p<0.001$), and with region x age ($F_{(5,80)}=23.11, p<0.001$) being observed. Primary WGE neurones expressed a greater percentage of GABA at all ages, with primary CTX neurones expressing the least, apart from at E20 where VM cells expressed fewer GABAergic neurones (Fig. 3.3b). (NB Glutamate, the main neurotransmitter for CTX cells was not examined in this study because CTX cells were only used as control group for proliferation and differentiation tests).
Figure 3.3 Percentage TH (a) and GABA (b) neurones generated from primary VM, WGE & CTX cells at different ages. Only VM cells generated TH positive neurones. A significant effect of age on TH expression was observed (p<0.01). Although GABAergic neurones were generated by primary cells from all regions, a significant difference in neurones from different regions expressing GABA was observed (p<0.001). Data expressed as mean ± S.E.M. across three separate experiments.

**p<0.01 vs. E11, E16, E18 & E20.
3.3.4 Neurogenic potential of short-term cultured progenitor cells

Figure 3.4 shows the percentage of neurones generated by different aged progenitor cells isolated from three different brain regions after 7-10 days proliferation and subsequent 7 days differentiation (passage 1).

Progenitor cells from all three regions and at all ages generated neurones, with neuronal yield ranging from 10-25% (Fig. 3.4a). A significant difference in the percentage of neurones generated by progenitors from different regions was observed ($F_{(2,219)}=52.85$, $p<0.001$), with VM progenitor cells generating fewer neurones compared to WGE and CTX progenitors at most ages. Progenitor cells isolated from the CTX generated the most neurones, except at E14 & E16 where neurogenesis of WGE progenitors was greater (Fig. 3.4a).

A significant difference of age on the neurogenic capacity of progenitors was also observed ($F_{(2,219)}=9.91$, $p<0.001$). Peak neurogenesis of VM progenitors was significantly higher at E14 than at E13 ($p<0.05$), and all other ages ($p<0.01$, Fig. 3.4a & 3.4b). WGE progenitors showed significantly higher neurogenesis at E14-16 when compared to all other ages ($p<0.01$, Fig. 3.4a & 3.4c), however, little difference was observed with CTX progenitors, with all ages generating similar percentage of neurones (Fig. 3.4a & 3.4d).

When compared to primary cell data, a significant reduction in neurogenesis for all cultured progenitors was observed ($F_{(1,448)}=5944.88$, $p<0.001$, Fig. 3.5a & b).
Figure 3.4 Neurogenic properties of different aged progenitor cells isolated from three different brain regions (VM, WGE & CTX), following 7-10 days proliferation (a). Both a significant difference in region and embryonic age on neuronal yield is observed (p<0.001 & p<0.001 respectively). Individual graphs showing the neurogenic properties of different aged progenitor cells isolated from the VM (b), WGE (c) & CTX (d), following 7-10 days proliferation are also shown. VM progenitors generate higher numbers of neurones at ages E13-14. Peak neurogenesis for WGE progenitors occurs at E14-16. No significant peak in neurogenesis is observed for progenitors isolated from the CTX. Data expressed as mean ± S.E.M. across three separate experiments. **a p<0.01 vs. all ages except E13, *b p<0.05 vs. E13, **c p<0.01 vs. all ages except E16, **d p<0.01 vs. all ages except E14.
Figure 3.5 A significant difference in the neurogenic potential of primary cells (a) and cultured progenitor cells (b) is clearly observed (p<0.001). Data expressed as mean ± S.E.M. across three separate experiments.
3.3.5 Generation of TH positive and GABAergic neurones from cultured progenitor cells

After 7-10 days proliferation and 7 days differentiation (passage 1), progenitor cells isolated from the VM, WGE & CTX failed to generate any neurones expressing TH. Neurones did show the expression of GABA, however, the percentage of GABAergic neurones was fewer than 5% for all regions and ages (Fig. 3.6).

Because of the relatively low numbers of GABAergic expression, there was no significant difference in the amount of GABAergic neurones generated by progenitor cells from different regions (F(2,78)=0.78, n.s), or at different ages F(3,78)=0.64, n.s).

Figure 3.6 Percentage of GABAergic neurones generated from progenitors following 7-10 days in culture. Fewer than 5% of neurones expressed GABA for all regions and ages. No significant difference in the numbers of GABAergic neurones generated by progenitors from different regions or across the age range is observed. Data expressed as mean ± S.E.M. across three separate experiments.
3.3.6 Neurogenic potential of long-term cultured progenitor cells

Figure 3.7 shows the effect of in vitro proliferation of E12, E14, E16 & E20 rat VM, WGE & CTX progenitor cells for up to 40 days (passage 5). The behaviour of primary and short-term cultured (passage one) progenitor cells has been described previously (section 3.3.2 & 3.3.4), so only passage 3 and passage 5 data will be discussed here.

Following 21-25 days proliferation and subsequent differentiation (passage 3), progenitor cells isolated from the VM, WGE & CTX generated less than 10% neurones (Fig 3.7c). WGE & CTX progenitors generated similar percentages of neurones, except at E20 where neurogenesis of CTX progenitors was greater. VM progenitors again generated lower number of neurones when compared to progenitors from the WGE & CTX (Fig. 3.7c).

The neurogenic capacity of VM & CTX progenitors was not affected by age, with progenitors generating constant percentages of neurones across the four ages. An effect of age was however observed for WGE progenitors with a decline in neurogenesis at E20 (Fig. 3.7c).

After 35-40 days proliferation (passage 5), progenitor cells from all regions generated less than 2% neurones (Fig. 3.7d). At E12 & E14, VM progenitors generated the lowest percentage of neurones, with WGE progenitors generating the most. At E16 & E20 however, most neurones were generated by progenitors isolated from the CTX.

Neurogenesis of CTX progenitors remained stable over the age range with no change in neurogenesis. WGE progenitors showed decreased neurogenesis at E16 & E20, with VM progenitors showing decreased neurogenesis at E20 (Fig. 3.7d).

Statistical comparison of all of the data showed an effect of region ($F(2,497) = 33.60$, $p<0.001$), age ($F(3,497) = 23.53$, $p<0.001$) and proliferation ($F(3,497) = 2716.86$, $p<0.001$) on the percentage of neurones generated by progenitor cells.

The decline in neurogenesis for progenitors from all regions following culturing is highlighted in Fig. 3.8a-c. The rate of neurogenesis for VM progenitors at E12, E14 & E16 was similar, however, E20 VM progenitors showed a reduced rate of neurogenesis at passage three (21-25 days proliferation) when compared to the other ages (Fig. 3.8a). WGE progenitors at E12, E14 & E20 showed similar rates of neurogenesis, with E16 progenitors showing slightly higher rates at passage five (Fig. 3.8b). No difference in the rate of neurogenesis was observed with CTX progenitors at all ages (Fig. 3.8c).
Figure 3.7 Neurogenic potential of primary (a) and 7-10 day (a), 21-25 day (c) & 35-40 day (d) proliferated progenitor cells, isolated from the VM, WGE & CTX. A significant decline in neurogenesis is observed following the culturing of progenitor cells from all regions (p<0.001). Data expressed as mean ± S.E.M. across three separate experiments.
Figure 3.8 Rate of neurogenesis for progenitors isolated from the VM (a), WGE (b) & CTX (c). The decrease in neurogenesis following time in culture is clearly observed for all progenitors.
3.3.7 Percentage Neurones vs. Total neurones

During cell analysis, it was apparent that the number of cells on the coverslips varied with age. To see whether this had any significance on the results, the percentage neuronal yield (percentage of 250-300 viable cells counted that were neurones) was compared to total neuronal yield (calculated from the total number of viable cells per culture) (Fig. 3.9-3.11). Since the percentage of neurones derived from primary VM, WGE & CTX cells have been discussed previously (section 3.3.2) only total neurone data will be discussed here.

Similar to the percentage neuronal yield of VM cells (see section 3.3.2), the total number of neurones derived from VM cells showed an age dependent effect ($F_{(6,55)}=47.15$, $p<0.001$). However, unlike the percentage neuronal yield data where peak neuronal expression for VM cells was observed at E14, total neuronal counts showed that E12 VM cells generated a significantly higher neuronal yield compared to all other ages ($p<0.01$), with neuronal yield gradually declining at E13 & E14, and a large decline at E16 being observed (Fig. 3.9a). In addition, the total number of neurones derived from E11, E13 & E14 were not significantly different from each other, but were significantly higher than the neuronal yield from cells at E16-E20, ($p<0.01$, Fig. 3.9a).

Data showing the percentage of TH positive neurones generated from VM cells is described in section 3.3.3. Similar to this data, the total number of TH positive neurones derived from VM cells also showed a significant effect of age ($F_{(6,55)}=81.76$, $p<0.001$). Significantly more neurones expressing TH, were however derived from E12 VM cells when compared to cells derived from E13 & E14 (NK: $t_{(6,2)}=5.298$, $p<0.05$) & (NK: $t_{(6,3)}=5.978$, $p<0.05$), which is in contrast to the percentage TH data where cells from E12-E14 showed peak TH expression.

No significant difference in number of neurones expressing TH at E13 and E14 was observed (NK: $t_{(6,2)}=0.680$, n.s), and primary cells from E11, E16, E18 & E20 derived significantly less TH neurones when compared to progenitors from all other ages ($p<0.01$, Fig. 3.9b).

When counting total neurones derived from WGE cells, a significant variation in neuronal numbers generated by primary cells from different ages was observed
(F(6,41)=9.12, p<0.001). Primary cells from E16 generated greater numbers of neurones than at all other ages, with the amount of neurones declining at E18 & E20 (Fig. 3.10a). This is in contrast to the percentage neuronal yield data, where peak neuronal expression was observed at E14-18.

The total number of GABAergic neurones generated by primary WGE cells is shown in Fig 3.10b. Like percentage WGE data (see 3.3.3), the total number of GABAergic neurones generated by WGE cells varies significantly with age (F(3,23)=13.93, P<0.001), with peak GABAergic expression occurring at E16, and the lowest number of GABAergic neurones being generated at E20 (Fig. 3.10b). Analysis of the data shows that GABAergic expression at E16 is significantly higher compared to expression at E12 (NK: t(3,3) = 10.8, p<0.01), E14 (NK: t(3,2)=9.272, p<0.01) & E20 (NK: t(3,4)=13.435, p<0.01).

Figure 3.11a shows the total number of neurones generated by primary cells isolated from the CTX. Similar to the data showing the percentage of neurones generated by CTX cells, the total number of neurones showed significant variation with age (F(6,41)=5.07, p<0.001). However, despite the percentage neuronal data showing peak neuronal yield at E14-18 (see section 3.3.2), the data from total number of neurones shows that the neuronal yield remains relatively stable across the ages of E11–E16, with a reduction in neurones at E18 & E20 being observed (Fig. 3.11a).

In addition, while the percentage GABAergic neurones generated by primary CTX cells shows no significant effect of age, the total number of neurones expressing GABA does; (F(3,23)=3.7, p<0.05), with GABA expression peaking at E14 (Fig. 3.11b).
Figure 3.9 Graph showing the total neuronal yield (a), and the total number of TH positive neurones (b), derived from primary cells isolated from the VM at different ages. A significant difference of age on both the total number of neurones and TH positive neurones derived from VM cells is observed (p<0.001). Data expressed as mean ± S.E.M. across three separate experiments. **a p<0.01 vs. all other ages, **b p<0.01 vs. E16, E18 & E20, *a p<0.05 vs. E13, *b p<0.05 vs. E14, **c p<0.01 vs. E11, E16, E18 & E20.
Figure 3.10 Graph showing the total neuronal yield (a), and the total number of GABA positive neurones (b), derived from primary cells isolated from the WGE at different ages. A significant difference of age on both the total number of neurones and GABA positive neurones derived from VM cells is observed ($p<0.001$). Data expressed as mean ± S.E.M. across three separate experiments. **$p<0.01$ vs. all other ages.
Figure 3.11 Graph showing the total neuronal yield (a), and the total number of GABA positive neurones (b), derived from primary cells isolated from the CTX at different ages. A significant difference of age on both the total number of neurones (p<0.001) and GABA positive neurones (p<0.05) derived from VM cells is observed. Data expressed as mean ± S.E.M. across three separate experiments.
3.4 Discussion

All primary cells isolated from the developing rat VM, WGE and CTX, at gestational ages ranging from E11 to E20 showed the expression of the neuronal marker β-tubulin-III. Progenitor cells expanded from these regions and ages also showed the generation of neurones, even when progenitors were expanded for up to 40 days. The degree of neurogenesis from these progenitors however varied considerably, with region, progenitor age, proliferation time in vitro and progenitor origin all having a significant effect on neuronal yield.

Although primary cells from all regions and ages showed neuronal yields ranging from ~50-70%, this probably reflects the proportion of cells that have pre-differentiated into neurones at the time of dissection, rather than the percentage of neurones generated from progenitor cells in vitro. Because of this reason and because these cells did not undergo periods of proliferation, these primary cells cannot be compared to expanded progenitor cells and will therefore be discussed separately. The neuronal yield of primary neurones isolated from the VM, WGE & CTX at different ages does however provide a baseline to which we can measure (but not directly compare) the potential of progenitor cells.

3.4.1 Differentiation of progenitor cells

Although the generation of neurones was seen from expanded progenitors isolated from the VM, WGE & CTX at different ages, not all cells differentiated into neuronal phenotypes. Results show that the maximum percentage of neurones were generated from E14 WGE 7-day expanded progenitors, where ~25% of all cells were neurones, indicating that 75% of cells were of non-neuronal phenotypes. Although not identified in this study, the remaining cell types probably consisted of a mixture of oligodendrocytes and astrocytes, with a possibility of some cells being non-differentiated, uncommitted progenitor cells. Evidence for glial differentiation of expanded progenitors has recently been shown in progenitors from the VM (O'keeffe & Sullivan., 2004). In this report, differentiated progenitor cells isolated from ages E12-E14 showed to be immunopositive for both GFAP & myelin basic protein (MBP) indicating the presence of astrocytes and oligodendrocytes respectively. Interestingly, both the expression of astrocytes and oligodendrocytes was shown to increase with progenitor age. The presence of nestin-positive cells reported in this study also
indicates, and supports our suggestion that undifferentiated cells are also contained within the medley of differentiated cell types. The presence of oligodendrocytes and astrocytes following differentiation of expanded progenitor cells has not only been shown for VM tissue, but differentiation of progenitors down a glial pathway has also been demonstrated for striatal progenitors (Svendsen et al., 1995; Ostenfeld et al., 2002). From these reports and others (see reviews Gage et al., 1995a; McKay, 1997), we are confident that the majority of non-neuronal cells seen in our cultures are a mixture of oligodendrocytes, astrocytes and undifferentiated cells.

The range of different cell types that have been reported to be generated from these progenitors illustrates the multipotency of these progenitors and suggests that progenitors may contain both unipolar progenitors that generate only neurones and bipolar progenitors that are capable of generating neurones and glia (Kilpatrick & Bartlett, 1993; Vescovi et al., 1993).

3.4.2 Regional specification of progenitor cells

Primary and progenitor cells isolated from the VM, WGE & CTX all showed different expressions of the neuronal marker β-tubulin, with WGE cells generating the most neurones in primary cultures and CTX progenitor cells generally generating the most neurones at passage one. Interesting progenitors isolated from the VM generated the least number of neurones in both primary and passage one cultures. This regional difference in neuronal expression from primary cells and progenitors from different regions indicate that these progenitors still keep some of their regional properties i.e. they show regional specification.

Although this pattern of neurogenesis from VM, WGE & CTX progenitors has been reported previously for cultured E14 rat progenitors (Ostenfeld et al., 2002), the data presented in this study shows that this pattern of neurogenesis is consistent for expanded progenitors isolated from ages ranging from E11-E20.

In the data reported here, the percentage of neurones generated by E14 rat WGE and CTX progenitors following 7-10 days proliferation is similar to earlier reports (Ostenfeld et al., 2002), however, VM progenitors generated more neurones than previously observed (Ostenfeld et al., 2002; O’Keeffe & Sullivan, 2004). This difference in the percentage of neurones generated by VM progenitors may be because of the difference in the passaging technique, or it may be down to culture
conditions used in the different studies. Our passaging regime meant that cells were only passaged when fully confluent or when neurospheres reached a pre-defined size, rather than at fixed intervals as in the reports by Ostenfeld et al., (2002) and O’Keeffe & Sullivan., (2004). This not only allowed sufficient cell-cell contact which is important when proliferating progenitor cells *in vitro*, but it also prevented neurospheres becoming too large, and thus stopped cells in the core of the neurospheres dying from the lack of media contact. Passaging cells as soon as they were confluent also prevented cell stress due to ‘overcrowding’ of proliferation flasks. Although the studies by Ostenfeld and O’Keeffe and others (Armstrong et al., 2003; Svendsen et al., 1995, 1997b; Li et al., 2005), have used both EGF & bFGF in the proliferation of progenitor cells, the progenitor cells used in this study were proliferated in the presence of bFGF alone. The absence of EGF in our culture conditions however, would not have been responsible for the increase of VM neurones since EGF has shown not to have beneficial effects on the differentiation of these cells (Smith et al., 2003).

The rational for using bFGF alone in this experiment was based on two main reasons. Firstly, progenitor expansion by bFGF alone is sufficient, with differentiation of progenitors being comparable to the differentiation of progenitors expanded in EGF & bFGF, as shown in our previous study (Smith et al., 2003). Secondly, EGF may not be the optimal mitogen to use when looking at neurogenesis since its use has been suggested to favour glial cell differentiation (McKay., 1997).

Evidence for the involvement of EGF in promoting gliogenesis is shown in the adult rat brain where infusion of EGF into the brain showed a decrease in the number of newborn neurones, and an increase in the generation of astrocytes migrating to the olfactory bulb. Infusions of bFGF on the other hand, resulted in an increase in newborn olfactory bulb neurones being observed (Kuhn et al., 1997). Further support for the role of EGF in glial differentiation comes from clonal analysis studies where EGF was shown to stimulate only glial lineage restricted cells, whereas bFGF showed stimulation of multipotent progenitors capable of generating neurones and astrocytes as well as these glial restricted progenitors (Kilpatrick & Bartlett., 1995).

The reasons for the regional differences in neurogenesis observed in this study are unclear, but perhaps it’s due to the composition of progenitors isolated from different
regions. With the existence of different progenitor populations that give rise to specific cell types (Kilpatrick & Bartlett, 1995; Vescovi et al., 1993), perhaps progenitors from the WGE contain more unipolar progenitors than bipolar progenitors, whereas VM progenitors may primarily consist of the bipolar type, thus explaining why VM tissue generates less neurones. Alternatively, the difference in neurogenesis may be because the culture conditions used were more favourable for WGE progenitors rather than for VM progenitors. Interestingly, proliferation of VM progenitors at lower oxygen levels has shown to be greater when compared to higher oxygen levels; with progenitors from the forebrain showing no difference in proliferation (Storch et al., 2001). Proliferation of VM progenitors in lower oxygen has also showed increased neurogenesis (Studer et al., 2000) indicating that VM progenitors are highly sensitive to oxygen levels.

The oxygen levels in our study may therefore have been too high for optimal VM proliferation and differentiation to occur, whereas WGE progenitors may have been unaffected, similar to the forebrain progenitors seen in the study by Storch, thus contributing to the regional variation in neurogenesis seen in the data reported here.

The regional differences are also highlighted by looking at the phenotypic identity of the neurones generated by primary cells from each region, with only cells from the VM generating neurones immunopositive for TH. TH immunopositivity of VM progenitors is expected since this is where dopaminergic neurones of the substantia nigra originate. No evidence of TH staining was observed in WGE or CTX cell cultures, unlike previous reports where progenitors from the cortex have been shown to contain cells immunopositive for TH (Roybon et al., 2004). The level of TH expression observed with VM progenitors was dependent on the progenitor age, thus emphasising the importance of gestational age of progenitors.

Primary cells from all regions showed evidence of GABA expression, but this again varied with region, with neurones from the WGE showing the highest expression and neurones from the CTX showing the least. Following 7-10 days proliferation, GABA expression was still evident by progenitors from all regions but no neurones generated by VM progenitors at any age were immunopositive for TH. This reduction in TH expression is consistent with previous reports (Yan et al., 2001; Ostenfeld et al.,
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2002; O'Keeffe & Sullivan., 2004), and is thought to be because of the restricted proliferation of dopamine neuroblasts (Ostenfeld et al., 2002). This failure of VM progenitors to differentiate into dopamine neurones is of great concern because if progenitors cannot generate sufficient numbers of dopamine neurones even after short-term expansion, then their use as a replacement for foetal tissue in neuronal replacement therapies is futile. Perhaps VM progenitors require exposure to a range or combination of factors/signals rather than to bFGF/EGF alone, as shown by Storch et al., (2001), where human mesencephalic tissue expanded for several months was shown to generate dopaminergic neurones following differentiation in the presence of interleukin-1b, interleukin-11, LIF and GDNF.

3.4.3 Effect of age on neuronal differentiation

The data reported in this chapter shows significant variation in the neuronal expression of primary and progenitor cells with age. This variation is particularly apparent when looking at primary cell data, where WGE & CTX progenitors show peak neuronal expression at E14-18, and VM progenitors at E14 (Fig. 3.2a-d). Although less neurones are generated in passage one cultures (see section 3.3.4), this pattern of neurogenesis with age is still apparent, particularly for WGE and VM progenitors (Fig. 3.4a-d).

Similar observations showing the different effects of progenitor age on neural differentiation have recently been reported in a smaller study by O'Keeffe & Sullivan (2004). In this report, a difference in neural differentiation of progenitors isolated from the VM at ages E12 to E14 was observed (O'Keeffe & Sullivan., 2004). Taken together, both the study by O'Keeffe and the data reported here illustrate the importance of progenitor age on neurogenesis and in selecting the appropriate progenitor age when investigating the neural potential of rat neural progenitor cells.

The difference in the degree of neuronal expression with age is probably related to the timing of neural development of the different brain regions. Neurogenesis in the VM has been reported to occur between ages E12 and E15 (Ostenfeld et al., 2002; Altman & Bayer., 1981; Marchand & Poirier., 1983), with neurogenesis in the WGE occurring between E12 to E22 (Ostenfeld et al., 2002; Fricker-gates et al., 2004) and neurogenesis in the CTX occurring between E13.5 to E19.5 (Ostenfeld et al., 2002).
Within these neurogenic time windows, regulation of neurogenesis must occur to account for the variation of neurogenesis that has been observed in this study. The mechanisms involved in this regulation are unknown, but perhaps progenitors respond to regional environmental cues to up- or down-regulate neurone production via cell mitosis. During the developmental maturation of particular brain regions, high requirements of neurones may be needed, so progenitor cells may undergo several rounds of mitosis at particular ages to generate more progenitor cells with the potential to generate neurones. For the WGE, this developmental maturation may occur between E14-18, whilst for the VM it is at E14, thus explaining the peaks of neurogenesis. Birth dating studies using BrdU have shown increased generation of striatal neurones at E14-18 (Fricker-gates et al., 2004), which is consistent with our data. Altman & Bayer (1981) used tritiated thymidine to show that VM neurones are born between E13-15, and Sinclair et al have shown a decrease in neurogenesis at E16 (Sinclair et al., 1999), this again is similar to the results observed in our study.

After these periods of development, and towards the end of the neurogenic time window, the full complement of neurones are probably present resulting in progenitors undergoing their final mitotic division, which means fewer progenitors, and less neurogenesis thus explaining why by E20, less neurones are generally observed.

Evidence of differing degrees of mitosis with age has been observed with VM progenitors where neurospheres generated from early aged VM progenitors are larger and contain more cells than neurospheres generated by older progenitors (O'Keefe & Sullivan., 2004). Analysis of our data showed similar behaviour, with more cells present in cultures derived from younger aged VM progenitors compared to older progenitors. Differing cell numbers were also observed in WGE & CTX cultures of different ages, indicating that mitosis might explain the variation in neurogenesis with progenitor age. This difference in cell number must be due to the mitotic nature of the progenitors and not a result of the cell plating procedure since a fixed number of cells (50,000 cells/coverslip) were plated for all progenitors from all ages.

Interestingly, although the report by O'Keeffe and ourselves indicate a difference in neural differentiation with age, a difference in the percentage of neurones generated by VM progenitors is observed between the two reports. O'Keeffe report that progenitor cells from E12 VM generate more neurones than progenitors from E14 VM, we however, show more neurones from E14 than E12. This difference is
probably due to the way results are analysed. In this study, the percentage neurones generated was calculated by counting the percentage of 250-300 viable cells that were neurones. O'Keeffe however, counted the number of neurones generated from total number of cells. Because of the variation in cell numbers with progenitor age, counting the total number of neurones or calculating the percentage number of neurones from total cells and not a proportion of the cells will provide a more accurate measurement of the neurogenic capacity of neural progenitor cells.

To make our results more meaningful, and more representative, the total numbers of neurones from primary progenitor cells from all three regions and all ages were counted. Comparison between the percentage and total neurones generated by the cells shows marked differences (Fig. 3.9-3.11), thus highlighting the difference between the methods of analysis. Interestingly, numbers of neurones and TH neurones generated by primary VM progenitors were higher at E12 than at E14 when represented as total numbers (Fig. 3.9a). E12 VM progenitors following 7-10 days proliferation also showed to generate more neurones than E14 progenitors (data not shown), consistent with the results observed by O’Keeffe and Sullivan.

The observation of E12 VM progenitors generating more neurones and in particular TH neurones is of great importance, especially when considering transplantation studies involving the grafting of VM cells into the brain. Most of these studies have used VM progenitors isolated from embryo’s aged E14 or older and have shown low survival rates (3-10%) of TH cells after transplanting into the brain (Olanow et al., 1996; Dunnett., 1992; Roybon et al., 2004). Since progenitors from E12 VM generate more neurones and importantly more TH neurones than E14 VM cells, we propose that the use of E12 VM tissue will result in more surviving TH cells and thus provide better functional grafts than those produced by E14 VM tissue.

3.4.4 Effect of in vitro proliferation on neurogenesis

Although progenitors from all regions of the embryonic rat brain continued to generate neurones up to passage 5 (~40 days), this continued proliferation of progenitor cells resulted in a significant reduction in neurogenesis. Proliferation of progenitors resulted in a neuronal yield of 2% by passage 5, compared to ~25% at passage 1. This reduction in neurogenesis is also observed when looking at the total number of neurones (data not shown). This loss of neurogenic potential is consistent
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with our previous finding (Smith et al., 2003), and although the precise reason for this decrease in neurogenesis is unknown, a number of reasons have been suggested. Perhaps the culture conditions may be missing essential factors causing a failure of the progenitors to follow a neuronal lineage. This deficiency in differentiation conditions has been the suggested reason for the restriction in the differentiation potential for expanded progenitors isolated from the mouse LGE (Skogh et al., 2003). Another possibility is that over time, progenitors undergo a specific asymmetric division called the 'switch point' at which progenitors down regulate neurone production and increase glial generation (Qian et al., 2000). Alternatively, progenitors destined to become neurones may be more vulnerable than non-neuronal progenitors and are therefore lost during the passaging regime. Because the method of cell dissociation is relatively severe, cell-cell contact is disrupted and cells are stripped of their receptors causing cellular trauma and probably cell death thus resulting in an overall decrease in neurogenesis with increased passaging. Dissociation of cells using the 'chopping' method described by Svendsen et al., (1998) will be less traumatic to the cells and may increase the neurogenesis of these progenitors. Whatever the reason may be, its clear from the data that progenitors isolated from the optimal age of tissue based on primary cell data are unable to make significantly higher neurones after proliferation. This failure to retain the ability to generate large number of neurones after periods in vitro is a big concern and this issue needs to be addressed if stem cells are to be a valuable tool in supplying a source of neurones for transplantation.

In summary, the data reported here show that the neurogenic capacity of primary and progenitor cells isolated from the developing brain is dependent on both region and age. Regional variations in the neurogenic potential of progenitors are observed, with progenitors from the WGE generally generating more neurones than progenitors from the VM and CTX. More importantly however, the gestational age at which primary and progenitor cells are isolated from the brain has a significant effect on neurogenic capacity of progenitors. Selecting progenitor cells of the appropriate gestational age is therefore crucial when investigating the characteristics of these progenitors. Since primary and progenitor cells from E12 VM generate more neurones and importantly more TH neurones (primary cells) than E14 VM cells, we propose that the use of E12 VM tissue in transplantation experiments will result in more surviving TH cells and
thus provide better functional grafts than those produced by E14 VM cells, which is the standard age used at present.

Another important finding is that the ability of progenitors to generate neurones is severely reduced following short periods of proliferation. This is of major concern and unless this problem is addressed, this may jeopardise the use of stem cells as a potential source of neurones for transplantation.
Chapter Four

Experiment Two - Effect of ascorbic acid on the yield of dopaminergic neurones

Summary
This chapter discusses whether pre-treating mesencephalic cells with the antioxidant, ascorbic acid, can increase the numbers of dopamine neurones both in vitro and in vivo.

Primary E14 rat mesencephalic cells were differentiated for 7 days in media containing different concentrations of ascorbic acid, and the percentage of surviving dopamine neurones were counted. At concentrations ranging from 20-100μM, a significant increase in the percentage of neurones expressing TH was observed when compared to controls, but at higher concentrations (500μM and above), ascorbic acid-induced neurotoxicity was evident.

Mesencephalic cell suspensions supplemented with 100μM ascorbic acid were also transplanted into unilateral 6-OHDA lesioned rats, and behavioural rotation was assessed 2, 4 & 6 weeks post-transplantation. Although grafts pre-treated with ascorbic acid contained more surviving dopamine neurones compared to non-treated grafts, no significant difference in rotation score was observed, with both groups showing a reversal of rotational bias. Rotational behaviour is therefore a sensitive test for graft functionality, but suffers ceiling effects that limit the ability to discriminate functional differences associated with graft size.

The increased number of dopamine neurones observed following ascorbic acid treatment is likely to reflect a selective survival effect. However, the possibility of ascorbic acid increasing the number dopamine neurones by stimulating dopaminergic differentiation cannot be excluded.
Chapter Four

4.1 Introduction

Transplantation of embryonic mesencephalic tissue into the dopamine deleted striatum has shown clear functional improvements by ameliorating behavioural deficits in a variety of animal models (Brundin et al., 1986; Low et al., 1987; Sirinathsinghji et al., 1990; Annett et al., 1994; 1997). The extent of functional recovery is however limited, with grafts unable to reverse all lesion-induced behavioural abnormalities. This partial recovery has been documented in rats, with grafts showing a reduction in rotation asymmetry but no improvement in neglect and grasping ability of the contralateral paw in food retrieval tasks (Dunnett et al., 1987). Grafts of embryonic dopamine neurones in unilateral 6-OHDA lesioned monkeys have also shown the attenuation of rotational bias, but no attenuation of ipsilateral head orientation, ipsilateral hand preference and neglect of contralateral stimuli (Annett et al., 1994). This limited recovery may in part relate to poor graft survival (only 5-10% of dopamine cells typically survive transplantation, Dunnett., 1992) since the degree of functional recovery is dependent on the number of surviving dopamine neurones in the grafts (Brundin et al., 1994; Kordower et al., 1997). The low survival of dopamine neurones following transplantation means that for sufficient functional benefits to be achieved in Parkinson’s disease patients, transplantation of mesencephalic tissue from 4-8 human embryos is required per side of the brain (Brundin et al., 2000). This requirement for large quantities of human embryonic tissue for each patient makes widespread clinical delivery impractical; therefore, increasing dopamine graft survival needs to be achieved if neuronal replacement therapies are going to succeed (Brundin et al., 2000).

The mechanisms involved in dopamine cell death have been shown to be mediated through apoptosis (Mahalik et al., 1994) with the majority of dopamine cells dying rapidly following cell isolation and preparation. Time course studies have shown that significant dopamine cell death occurs over the first 24 hours following cell dissociation (Zawada et al., 1998), and in particular the first 8 hours where 50% of all dopamine cells die (Branton & Clarke., 1999). Grafted dopamine neurones have also been shown to die rapidly with the majority of cell loss occurring over the first 7 days after transplantation (Barker et al., 1996; Brundin et al., 2000).
Although the specific reasons involved in the cell death of dopamine neurones are unclear, cell preparation methods have been shown to be very important, with the number of \textit{in vitro} and \textit{in vivo} dopamine neurones being influenced with trituration technique, trypsin type and incubation time in trypsin (Barker \textit{et al.}, 1995).

One possible reason for the poor survival of dopamine neurones may be because of oxidative stress caused by free radicals, a mechanism which has been implicated in the death of substantia nigra dopamine neurones in Parkinson's disease (Sian \textit{et al.}, 1994; Olanow., 1993; Fahn & Cohen., 1992). Strong evidence for the involvement of oxidative stress in dopamine cell death comes from studies showing decreased sensitivity to the neurotoxin MPTP following antioxidant treatment. Neurotoxicity of substantia nigra dopamine neurones, caused by free radicals generated by the metabolism of MPTP, is abolished in transgenic mice with increased superoxide dismutase activity (Przedborski \textit{et al.}, 1992). Similar observations have been observed in mice treated with the antioxidants \(\alpha\)-tocopherol, \(\beta\)-carotene, ascorbic acid or \(N\)-acetylcysteine prior to MPTP administration (Perry \textit{et al.}, 1985). In addition, in MPTP injected mice, glutathione (a naturally occurring antioxidant present in a variety of brain regions including the substantia nigra) (Sian \textit{et al.}, 1994), is depleted, however this depletion is prevented following antioxidant treatment (Yong \textit{et al.}, 1986). Together, these studies suggest that oxidative stress plays an important role in the pathogenesis of Parkinson's disease.

Similarly, the underlying cause for why dopaminergic neurones are vulnerable when either cultured or transplanted into the brain may also be because of oxidative stress. It is thought that oxidative stress caused by hypoxia and cellular trauma during dissection and tissue preparation is one of the mechanisms contributing to poor dopamine survival (Brundin \textit{et al.}, 2000). Removing cells from low oxygen environments and exposing them to standard oxygen levels of 20% may also promote oxidative stress and therefore decrease dopamine cell survival. This might explain why increased dopamine survival is observed when mesencephalic tissue is cultured in oxygen levels representative of those levels found in developing tissues (Colton \textit{et al.}, 1995; Studer \textit{et al.}, 2000; Storch \textit{et al.}, 2001).

The neuroprotective effects of antioxidants on isolated mesencephalic cells also provide convincing evidence that the cell death of dopamine neurones is mediated via
oxidative stress (Colton et al., 1995; Love et al., 2002). Supplementing mesencephalic cell suspensions with glutathione shows increased survival of dopamine neurones (Love et al., 2002). Conversely, inhibition of glutathione synthesis by using L-buthionine sulfoximine, results in a significant reduction in the survival of these cells (Grasbon-Frodl et al., 1996). Dopamine neurones in glutathione-depleted cultures can be rescued by treating cultures with lazaroids (Grasbon-Frodl et al., 1996). Lazaroids inhibit lipid peroxidation and the formation of free radicals, and have been also shown to increase dopamine survival in vitro (Frodl et al., 1994), as well as increasing cell viability of dissociated mesencephalic cells and importantly increasing dopamine survival in grafts (Nakao et al., 1994). The increased dopamine survival in grafts following lazaroid treatment is reflected in amelioration of rotational bias in unilateral lesioned rats, with faster recovery of rotational asymmetry being observed compared to rats receiving non-treated grafts (Nakao et al., 1994). Interestingly, grafts pre-treated with glutathione alone, do not show increased survival of dopamine neurones and attenuation of rotational behaviour when compared to controls (Love et al., 2002), however when glutathione is combined with ascorbic acid, an increase in dopamine survival and reduction in rotation asymmetry is apparent (Agrawal et al., 2004).

Because the majority of dopamine cell death occurs over the first 24 hours from cell dissociation and this coincides with rapid ascorbic acid depletion in cultured cells (Kalir & Mytilineou., 1991), we investigated whether the anti-oxidant effects of ascorbic acid alone, could increase dopamine survival in both cultured neurones and in grafts transplanted into the unilateral 6-OHDA lesioned rat brain.

E14 rat mesencephalic tissue was dissociated and differentiated in media containing different concentrations of ascorbic acid, and the number of neurones and TH positive neurones was assessed. Cells supplemented with ascorbic acid were also grafted into the unilateral 6-OHDA lesioned rat brain. The effect of grafts supplemented with ascorbic acid was compared to untreated grafts on rotational behaviour at 2, 4 & 6 weeks post-transplantation.

After behavioural testing, the numbers of dopamine neurones in grafts pre-treated with or without ascorbic acid were counted and compared.
4.2 Experimental Procedure

A detailed description of the dissection procedure and in vitro techniques used in this chapter can be found in chapter two, section 2.2.1, 2.2.2, & 2.6.

For cell viability tests, dissected VM tissue was dissociated (see chapter two, section 2.4) and incubated at 37°C, 5% CO\textsubscript{2} for 1 hour in HBSS supplemented with different concentrations of ascorbic acid. Following incubation, the viability of cells was assessed by counting cells while using trypan blue as an indicator of dead cells (see chapter two, section 2.6.1).

For in vitro experiments, dissociated cells were differentiated for 7 days in media consisting of neurobasal medium supplemented with 1% B27, 1% antibiotic/antimycotic solution, 30mM glucose, 1mM glutamine, 50ng/ml bovine serum albumin, 1% foetal calf serum and ascorbic acid ranging from 1-5000μM at 37°C & 5% CO\textsubscript{2}. Following 7 days differentiation, cells were fixed in 4% PFA and stained with the marker β-tubulin-III for the identification of neurones. Neurones were also stained for TH for the identification of dopamine neurones (see chapter two, section 2.6.2).

For in vivo experiments, adult female Sprague Dawley rats (~225g) received 6-OHDA lesions of the right medial forebrain bundle (see chapter two, section 2.7.1) and were rotated for 90 minutes following 2.5mg/kg amphetamine stimulation (see chapter two, section 2.8.1.1). Only rats showing good rotational behaviour (at least 600 net ipsilateral turns over 90 minutes) were used in this experiment. As a control, non-lesioned rats were also tested for rotation bias. Approximately 3 weeks after lesioning rats received transplants of either VM cells into the right striatum (n=9, group 1), VM cells with 100μM ascorbic acid (n=10, group 2) or 100μM ascorbic acid only (n=8, group 3). Group 4 (n=8) consisted of animals with unilateral lesions only, and group 5 (n=6) consisted of non-lesioned animals. All groups were rotated at 2, 4 & 6 weeks post-transplantation. After behavioural testing, animals were sacrificed, brains were isolated and the striatal dopamine grafts were analysed (see chapter two, section 2.9.4).

All in vitro experiments were repeated three times and statistical tests of analysis of variance (ANOVA) were analysed using the statistical package Genstat v7.2, with further analysis of the results being achieved by using the Neuman Keuls t-test.
4.3 Results

4.3.1 Cytotoxicity of ascorbic acid

To examine whether ascorbic acid had any cytotoxic effects, primary VM cells were incubated in ascorbic acid at concentrations ranging from 1μM-5mM. Following incubation for one hour at 37°C, cell viability was assessed using the trypan blue exclusion assay. Viable cells appeared phase bright and spherical when observed under the microscope, whereas damaged cells appeared blue.

The effects of ascorbic acid concentration on cell viability can be seen in figure 4.1. A significant effect of ascorbic acid concentration on cell toxicity was observed ($F_{(10,32)}=10.55$, $p<0.001$), with cells exposed to concentrations of 2500μM (2.5mM) & 5000μM (5mM) showing significantly increased cell death when compared to controls (~9% compared to 4%, $p<0.01$) and all other concentrations ($p<0.01$). Cells exposed to ascorbic acid concentrations ranging from 1-1000μM did not show any significant toxic effects when compared with control cultures (Fig. 4.1).

![Figure 4.1](image)

**Figure 4.1** Cytotoxicity of ascorbic acid. A significant effect of ascorbic acid concentration on cell death is observed ($p<0.001$), with cells exposed to concentrations of 2500μM & 5000μM showing significantly higher cell death rates than compared to all other concentrations ($p<0.01$). Cytotoxic effects of ascorbic acid at concentrations ranging from 1μM-1000μM are not significantly different to control (0μM). Data expressed as mean ± S.E.M. across three separate experiments.

**a** $p<0.01$ vs. all concentrations except 5000μM, **b** $p<0.01$ vs. all concentration except 2500μM.
4.3.2 Cell differentiation

Cells isolated from E14 rat ventral mesencephalic tissue were differentiated immediately following dissection in media containing different concentrations of ascorbic acid ranging from 1μM-5mM. After one day of differentiation, cells were observed to generate a variety of cell phenotypes as apparent by the presence of different cell morphologies. Although no obvious difference in cell differentiation was observed at low ascorbic acid concentrations (1-250μM), ascorbic acid-induced cell death was evident at higher doses (500-5000μM) since the establishment of cell cultures was not apparent. Following 7 days differentiation, neuronal phenotypes were identified using the neuronal marker β-tubulin-III and dopaminergic neurones were identified by examining neurones immunopositive for the antibody tyrosine hydroxylase (TH) (Fig. 4.2).
Figure 4.2 Fluorescent immunochemistry of β-tubulin-III positive cells and TH positive neurones. Neurones are stained using β-tubulin-III and visualised by green florescence. TH neurones are stained using TH and visualised by red fluorescence. All cell bodies are stained blue with Hoechst. More TH positive neurones are observed at 20μM and 100μM ascorbic acid when compared with controls (0μM). At 1000μM ascorbic acid, no neurones are evident. Scale bar = 100μm
4.3.3 Effect of ascorbic acid on neuronal yield

A significant effect of ascorbic acid concentration on neuronal yield was observed (F(7,104)=17.36, p<0.001), with cultures treated with 500μM ascorbic acid showing a significant reduction in the generation of neurones when compared to control cultures (42% vs. 69%, NK: t(7,8)=13.023, p<0.01), and concentrations of 1000-5000μM generating no neurones at all (p<0.01, Fig. 4.3). At ascorbic acid concentrations ranging from 1-250μM, there was no significant difference in neuronal expression when compared to controls, with neuronal yield ranging from 61-70%.

![Graph showing effect of ascorbic acid concentration on neurone survival. A significant effect of ascorbic acid concentration on neuronal generation is observed (p<0.01), with concentrations of 500μM and above significantly reducing the percentage of neurones generated (p<0.01). Data expressed as mean ± S.E.M. across three separate experiments. ** p<0.01 vs. 0 - 250μM.]

Figure 4.3 Effect of ascorbic acid concentration on neurone survival. A significant effect of ascorbic acid concentration on neuronal generation is observed (p<0.01), with concentrations of 500μM and above significantly reducing the percentage of neurones generated (p<0.01). Data expressed as mean ± S.E.M. across three separate experiments. ** p<0.01 vs. 0 - 250μM.
4.3.4 Effect of ascorbic acid on survival of dopamine neurones

In addition to examining the neuronal yield of primary VM cells, the effect of ascorbic acid concentration on the expression of TH positive neurones (presumed dopaminergic) was also assessed. A significant effect of ascorbic acid concentration on TH expression was observed \( (F_{(7,104)}=51.77, \ p<0.001) \), with cells exposed to concentrations of 20\( \mu \)M, 50\( \mu \)M, 100\( \mu \)M (\( p<0.01 \)) and 250\( \mu \)M (\( p<0.05 \)) generating significantly more TH immunopositive neurones when compared to controls (Fig. 4.2). TH expression at 20, 50 and 100\( \mu \)M was significantly higher than at all other concentrations, with no difference in TH expression between these three concentrations being observed (\( p<0.01 \)). Interestingly, cells treated with 500\( \mu \)M ascorbic acid generated significantly less TH positive neurones compared to all other conditions (\( p<0.01 \)). At low ascorbic acid concentrations of 1\( \mu \)M & 10\( \mu \)M, no significant difference in TH expression was observed compared to controls (Fig. 4.4).

![Figure 4.4](image)

**Figure 4.4** Effect of ascorbic acid concentration on dopamine neurones. A significant effect of ascorbic acid concentration on TH expression is observed (\( p<0.01 \)), with concentrations of 20\( \mu \)M, 50\( \mu \)M and 100\( \mu \)M ascorbic acid generating significantly higher TH expression compared to all other concentrations. Data expressed as mean ± S.E.M. across three separate experiments. **a** \( p<0.01 \) vs. controls and all other concentrations except 50 & 100\( \mu \)M, **b** \( p<0.01 \) vs. controls and all other concentrations except 20 & 100\( \mu \)M, **c** \( p<0.01 \) vs. controls and all concentrations except 20 & 50\( \mu \)M, **d** \( p<0.05 \) vs. controls.
4.3.5 VM grafts and amphetamine-induced rotations

To examine whether dopamine grafts treated with ascorbic acid could increase the number of surviving dopamine neurones in vivo, primary VM cells treated with 100mM ascorbic acid were grafted into unilateral 6-OHDA-lesioned rats, and rotational behaviour was assessed.

The effect of E14 rat VM grafts transplanted with and without 100μM ascorbic acid, on unilateral 6-OHDA lesion-induced rotational asymmetry can be seen in figure 4.5. Also shown is rotational behaviour of rats that received 100μM ascorbic acid only, and lesioned only and non-lesioned rats.

Rats receiving VM grafts only (n=9, group 1), showed no difference in rotational behaviour at 2 weeks post-transplantation compared to pre-graft scores (NK: t(2,4)=1.023, n.s). Significant decrease in rotational behaviour towards the ipsilateral side of the lesion at 4 weeks (NK: t(3,4)=16.442, p<0.01) and 6 weeks post-transplantation (NK: t(4,4)=17.098, p<0.01) were however observed, with the graft causing overcompensation of motor asymmetry resulting in rotations contralateral to the side of the lesion.

Similar behaviour was observed for rats receiving VM grafts + 100μM ascorbic acid (n=10, group 2). No significant difference in rotational behaviour was observed at 2 weeks post-transplantation when compared to pre-graft scores (NK: t(2,4)=1.112, n.s). Significant rotational behaviour was however observed at 4 (NK: t(3,4)=17.683, p<0.01) and 6 weeks (NK: t(4,4)=19.483, p<0.01) post-transplantation. No significant difference in rotational behaviour between the two VM grafted groups was observed at 4 weeks (NK: t(2,12)=0.138, n.s) or 6 weeks (NK: t(2,12)=0.711, n.s) post-transplantation.

Rats that received ascorbic acid only (n=10, group 3) and the lesion only group (n=10, group 4) showed stable rotations over the 6-week period, with no significant change in rotational behaviour. Non-lesioned rats (n=6, group 5), showed no significant rotational bias. Statistical analysis of the data confirms a significant difference in rotational behaviour with groups (F(4,167)=12.66, p<0.001). A significant difference in rotations with time is also observed (F(3,167)=15.23, p<0.001).
Figure 4.5 Amphetamine-induced rotational behaviour. A significant difference in rotational bias with groups is observed (p<0.001), with both the VM grafted and VM + ascorbic acid groups showing a reduction in rotation at 4 and 6 weeks when compared to pre-graft rotation scores. No significant difference is however observed between these two groups. Lesion only and ascorbic acid only groups show no significant difference in rotational behaviour. Non-lesioned rats show no significant rotational bias. **p<0.01 vs. pre-graft scores. Data expressed as mean ± S.E.M.
4.3.6 *Number of TH positive neurones in grafts*

Grafts of dopamine neurones into the dopamine depleted rat striatum can clearly be seen along with the characteristic halo of dopamine innervation (Fig. 4.6C). Grafts treated with or without ascorbic acid both contain numerous TH positive neurones which extensively project throughout the grafted site (Fig. 4.6D & E).

By using the Abercrombie correction formula (see chapter two, section 2.9.4), the number of grafted TH positive present in the each rat brain was calculated. Analysis of the data showed VM grafts with 100μM ascorbic acid contained significantly higher numbers of TH neurones when compared to grafts of VM cells only (p<0.05) (Fig. 4.7).
Figure 4.6 TH immunohistochemical staining in brain sections moving rostral-caudal from left to right through the levels of the striatum and the substantia nigra. Control groups show equal staining for TH in the striatum and substantia nigra on both sides of the brain (A). Lesion groups show clear depletion of TH staining on right hand side, in both the striatum and the substantia nigra (B). Scale bar represents 2.5 mm. Grafts can be clearly seen in the striatum (C). Grafts of VM tissue alone and VM + ascorbic acid show the presence of many TH neurones (D & E respectively). Scale bars in C = 1mm, D & E = 100μm.

Figure 4.7 Number of TH positive cells in grafts. Grafts of VM tissue transplanted in the presence of ascorbic acid show significantly more TH neurones than grafts of VM tissue alone. * p<0.05
4.4 Discussion

Transplantation of embryonic mesencephalic dopamine neurones into the brains of Parkinson’s disease patients has shown proof of the concept that neuronal replacement strategies are successful as clinical treatments (Lindvall et al., 1994). Although results from these trials are encouraging, with improvements in symptoms being observed, not all aspects of disease symptoms are ameliorated which is probably attributed to inadequacies in graft survival (Annett et al., 1997). For neuronal replacement strategies to succeed, increasing the number of surviving dopamine neurones in grafts is therefore an imperative requirement (Brundin & Bjorklund., 1998).

Because of the substantial experimental evidence implicating the involvement of oxidative stress in dopamine vulnerability (Frodl et al., 1994; Grasbon-Frodl et al., 1996; Nakao et al., 1994; Love et al., 2002), and evidence showing that the majority of transplanted dopamine cells die over the first 24 hours (Zawada et al., 1998) which is coincidently consistent with a rapid reduction in ascorbic acid levels (Kalir & Mytilineou., 1991), we investigated whether ascorbic acid could increase the survival of both cultured and transplanted dopamine neurones possibly by exerting its antioxidant effects and protecting against free radical damage.

4.4.1 Cytotoxicity of ascorbic acid

Before examining the effects of ascorbic acid on the survival of dopamine neurones, it was important to assess whether ascorbic acid had any cytotoxic effects on dissociated mesencephalic cells. Using the trypan blue exclusion assay, the percentage of dead cells were counted following incubation in ascorbic acid at concentrations ranging from 1-5000µM. Although no cytotoxicity was observed at low concentrations when compared to controls (Fig. 4.1), an increase in cell death was observed at ascorbic acid concentrations of 2500 & 5000µM. The precise reasons for this toxicity are unclear but perhaps the addition of large concentrations of ascorbic acid resulted in the cell environment being too acidic for cells to survive. Interestingly, severe toxicity was observed in neuronal cultures exposed to 500µM ascorbic acid (Fig. 4.3). At this concentration a significant reduction in the percentage of neurones was observed when compared to controls, with higher concentrations resulting in total neuron death. It is surprising that ascorbic acid at these concentrations has adverse effects on neurones since neuronal ascorbic acid levels in vivo are in the region of 10mM (Rice...
Perhaps the absence of buffering systems in the culture environment again resulted in an excessive acidic environment that neurones cannot tolerate. Alternatively, high concentrations of ascorbic acid may favour free radical formation and therefore oxidative stress of cultured neurones. Auto-oxidation of ascorbic acid can generate the superoxide free radical (Grünewald, 1993) and hydrogen peroxide (Clement et al., 2001) which damage critical biological molecules. Evidence for this has been shown in cultures of fibroblast cells where the addition of catalase (an enzyme that removes hydrogen peroxide) to fibroblast cells exposed to 300μM ascorbic acid prevents cell death (Arakawa et al., 1994). Signs of free radical-mediated damage caused by ascorbic acid have also been shown in vivo where lymphocyte DNA isolated from volunteers whose diets were supplemented with 500mg of vitamin C showed increased levels of 8-oxoadenine – a marker for free radical-mediated DNA damage (Podmore et al., 1998).

Together, these studies show that ascorbic acid can produce harmful products capable of damaging cells, and the cytotoxicity of cultured neurones observed in this study is probably due to its pro-oxidant properties. It is important to note that because of the presence of endogenous antioxidants which protect against such oxidative insults, it is unlikely that this toxicity would occur normally in vivo where the neuronal ascorbate concentrations are higher. The difference between the in vivo and in vitro environments means that extrapolating conclusions made from in vitro data and applying them to in vivo situations must therefore be done with caution.

4.4.2 Vulnerability of dopamine neurones

Although all cells are susceptible to free radical damage, dopamine neurones are particularly vulnerable because dopamine itself can be toxic (Hastings et al., 1996; Filloux & Townsend., 1993) through oxidative metabolism and the generation of free radicals. Similar to the neurotoxicity caused by 6-OHDA administration, where 6-OHDA is taken up by dopamine terminals and oxidised producing hydrogen peroxide ($H_2O_2$, Box 1, equation 1) and killing neurones from within (Cohen & Heikkila., 1974; Heikkila & Cohen., 1971; 1973), dopamine can also produce hydrogen peroxide following its metabolism by monoamine oxidase (Box 1, equation 2, Olanow., 1993; Dunnett & Björklund., 1999). Alternatively, dopamine can also undergo auto-oxidation to produce the superoxide radical ($O_2^-$), this in turn can react
with further dopamine producing hydrogen peroxide (Box 1, equation 3, Olanow., 1993; Dunnett & Björklund., 1999). Hydrogen peroxide itself is not excessively toxic and it is normally removed by glutathione (Olanow., 1993; Box 1, equation 4), however, hydrogen peroxide has the ability to generate harmful free radicals which makes its presence so dangerous. In environments where oxidative stress is favoured i.e. during dissection and preparation of tissue, hydrogen peroxide can interact with the superoxide radical and form the extremely reactive and toxic hydroxyl ion (*OH, Haber-Weiss reaction, Box 1, equation 5, Olanow., 1993). Hydrogen peroxide can also react with iron, again leading to the formation of harmful hydroxyl ions (Fenton reaction, Box 1, equation 6, Olanow., 1993; Dunnett & Björklund., 1999). These additional oxidative pathways increase the possibility of free radical formation and subsequently oxidative stress and therefore may explain why dopamine neurones are exceptionally vulnerable.

### Box 1. Formation of hydrogen peroxide and free radicals

1. Oxidation of 6-Hydroxydopamine
   \[ 6\text{-OHDA} + \text{O}_2 \rightarrow Q + \text{H}_2\text{O}_2 \]

2. Enzymatic metabolism of dopamine
   \[ \text{DA} + \text{O}_2 + \text{H}_2\text{O} \rightarrow 3,4\text{-DOPAA} + \text{NH}_3 + \text{H}_2\text{O}_2 \]

3. Auto-oxidation of dopamine
   \[ \text{DA} + \text{O}_2 \rightarrow \text{SQ} + \text{O}_2^- + \text{H}^+ \]
   \[ \text{DA} + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{SQ} + \text{H}_2\text{O}_2 \]

4. Clearance of H2O2
   \[ 2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \]

5. Haber-Weiss reaction
   \[ \text{H}_2\text{O}_2 + \text{O}_2^- \rightarrow \cdot\text{OH} + \text{OH}^- + \text{O}_2^- \]

6. Fenton reaction
   \[ \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \cdot\text{OH} + \text{OH}^- + \text{Fe}^{3+} \]

**Abbreviations:**
- 6-OHDA - 6-hydroxydopamine
- \text{H}_2\text{O}_2 - Hydrogen peroxide
- DA - Dopamine
- 3,4-DOPAA - 3,4 dihydroxyphenylacetaldehyde
- Q - Quinone
- SQ - Semiquinone
- \text{O}_2^- - Superoxide anion
- GSH - Glutathione
- GSSG - oxidised glutathione
4.4.3 Increased expression of dopamine neurones following treatment with ascorbic acid

Pre-treating cultured dopamine neurones with ascorbic acid showed a dose-dependant effect on the expression of dopamine neurones. At concentrations ranging from 20-100μM, dopamine neurone expression was significantly higher compared to controls with almost a 2-fold increase in dopamine expression being observed. This increase in dopamine neurones following ascorbic acid treatment is consistent with an earlier report that showed increases in dopamine and its primary metabolite 3,4 dihydroxyphenylacetic acid (DOPAC) in cultured mesencephalic neurones following treatment with the same anti-oxidant (Kalir & Mytilineou., 1991).

Transplanted cells pre-treated with 100μM ascorbic acid also showed a significant increase in the number of dopamine neurones when compared to non-treated grafts. Although increases in grafted dopamine neurones haven’t been reported following ascorbic acid treatment alone, similar observations have been reported following ascorbic acid and glutathione treatment (Agrawal et al., 2004).

Increased survival of dopamine neurones have also been shown in a number of previous studies following treatment with different anti-oxidants and lazaroids (Colton et al., 1995; Frodl et al., 1994; Love et al., 2002; Nakao et al., 1994), which suggests that ascorbic acid also has its effects by reducing the likelihood of oxidative stress.

However, although ascorbic acid is a major antioxidant, it also has a variety of other functions such as being involved in excitatory amino acid release, acting as co-factor in the synthesis of neuropeptides, stimulating myelin formation by Schwann cells and stimulating acetylcholine and noradrenaline release from synaptic vesicles (Rice., 2000; Grünwald., 1993; Kalir & Mytilineou., 1991). Because we did not assess oxidative stress mediated toxicity which can be measured by analysing the levels of certain markers such as 8-oxoadenine, 8-oxoguanine (Podmore et al., 1998), 8-hydroxyguanine and 8-hydroxydeoxyguanosine (Helbock et al., 1999) and coupled with the fact that ascorbic acid has multi-functional properties, the implication that ascorbic acid increased dopamine survival by reducing oxidative stress mechanisms would only be an assumption. The possibility that ascorbic acid increased dopamine expression through another mechanism, other than reducing oxidative stress, therefore cannot be excluded.
Interestingly, a previous report showed that the increase in dopamine neurones from proliferated mesencephalic progenitors treated with ascorbic acid showed no difference in the expression of markers for oxidative stress when compared to control cultures, nor did any other anti-oxidant mimic the effect of ascorbic acid. From this it was concluded that ascorbic acid exerts its effects not by acting as an anti-oxidant and increasing the survival of dopamine neurones, but rather by activating differentiation pathways and promoting the dopaminergic differentiation of neurones (Yan et al., 2001).

Increased differentiation as opposed to increased survival of dopamine neurones could also explain the increase in dopamine neurones observed in this study. It has been previously reported that the expression of dopamine neurones in rat cortical cell cultures was increased by exposing cells to dopamine and subsequent stimulation of D1 and D2 receptors (Zhou et al., 1996). Perhaps ascorbic acid-induced increases in dopaminergic expression is also mediated in the same way since ascorbic acid would increase the amount of dopamine by decreasing dopamine auto-oxidation and therefore allowing stimulation of D1 and D2 receptors.

4.4.4 Rotational behaviour is not useful for a measure of graft size

Determination of dopamine graft survival following pre-treatment with ascorbic acid was assessed by measuring rotational asymmetry in 6-OHDA lesioned rats. Prior to grafting, only rats displaying at least 600 ipsilateral turns following amphetamine stimulation over the 90-minute testing period were selected, which subsequently reflects near total depletion of striatal dopamine (Brundin et al., 1988). Even though grafts pre-treated with ascorbic acid contained more neurones than non-treated grafts, no difference in rotational bias between grafts groups was observed, with both groups showing attenuation of rotational behaviour by 6 weeks (Fig. 4.5). This is consistent with an earlier report that showed grafts pre-treated with lazaroids contained more dopamine neurones but showed no significant difference in rotation to control grafts 6 weeks post-transplantation (Nakao et al., 1994).

The reason why increased survival of dopamine neurones in pre-treated grafts is not reflected in rotational behaviour tests is probably because the control grafts probably contained enough dopamine cells to reach saturation point (Björklund et al., 1980; Brundin et al., 1988). At this point, no further increases in dopamine cells within
grafts will show any further effect on rotational behaviour (Brundin et al., 1988). Since this saturation point occurs when as few as 300-500 dopamine neurones are present, and the number of dopamine neurones in non-treated and treated grafts in our study showed in the region of 2000 dopamine neurones, it is unsurprising that no difference in rotational behaviour was observed. For this difference in graft size to be observed in vivo, less sensitive behavioural tasks should be employed such as the lateralised associative learning task recently used by Dowd & Dunnett., (2004).

In contrast to the data reported here, a recent report did show a significant difference in rotational behaviour between rats that received either grafts pre-treated with glutathione and ascorbic acid or non-treated grafts, even though both graft groups contained enough neurones to reach saturation point (Agrawal et al., 2004). Interestingly, this study also showed increased dopamine cells in the substantia nigra following grafts treated with antioxidants compared to non-treated grafts. No evidence of dopamine cells was observed in the substantia nigra in our study and its unlikely that grafts of dopamine neurones in the striatum would cause neurogenesis of dopamine neurones in the substantia nigra. Instead this apparent neurogenesis of dopaminergic nigral neurones reported by Agrawal is probably a result of insufficient lesioning of the dopamine pathway rather than reflecting possible neurogenesis. This insufficient lesioning of the dopamine pathway may explain why a difference in rotational bias was observed between both graft groups.

In conclusion, the data reported in this study shows that pre-treatment of mesencephalic neurones with ascorbic acid increased the number of dopamine neurones, both in culture and when transplanted into the brain. Although it is tempting to attribute the effects to the antioxidant properties of ascorbic acid, the possibility of ascorbic acid increasing dopaminergic expression via other mechanisms, for example, by enhancing dopaminergic differentiation, cannot be excluded. To be certain that antioxidant mechanism were involved, levels of oxidative stress markers should be measured.

However, while the precise mechanisms of remain elusive, increasing the yield of dopamine neurones by using ascorbic acid may improve the efficacy of transplantation studies.
Chapter Five

Experiment Three – Neurogenic potential of adult hippocampal neural progenitor cells

Summary

This chapter investigates the potential of adult hippocampal neural progenitor cells to differentiate into neurones of dopaminergic phenotypes following short-term in vitro proliferation.

Neural progenitor cells were isolated from the hippocampus of 6-week-old rats and cultured in media supplemented with bFGF for 21 days. Following proliferation, cells underwent 7 days differentiation and the generation of astrocytes, neurones and dopaminergic neurones was assessed by examining cells immunopositive for GFAP, β-tubulin-III and TH respectively.

Results demonstrate that adult neural progenitor cells derived from the hippocampus generate neurones and astrocytes, thus displaying their multipotent properties. However, the level of neurogenesis is extremely low and, more importantly, there was no evidence for the generation of dopamine neurones since no neurones were immunopositive for TH. In a second part of this study, transplantation of adult hippocampal progenitor cells into the developing ventral mesencephalon was planned, however, the proliferation of progenitors generated insufficient numbers of cells for transplantation.

This study shows that generating large numbers of cells from adult hippocampal neural progenitor cells and their differentiation into neurones, in particular dopamine neurones, is proving difficult and may explain why research in this area has gone relatively quiet over the last few years.
5.1 Introduction

The long-standing dogma that the brain is a structure incapable of generating new neurones has now been proved wrong with the discovery of continued neurogenesis in specific areas of the adult brain. Neural stem cells have shown to exist predominantly in two distinct brain regions, namely the anterior regions of the subventricular zone (SVZa), and the dentate gyrus of the hippocampus (Altman & Das., 1965; Gage et al., 1995; Kuhn & Svendsen., 1999; Seaberg & van der Kooy., 2002). Cells originating from the SVZ migrate along the rostral migratory stream into the olfactory bulb where they differentiate into dopaminergic and GABAergic interneurones (Lois & Alvarez-Buylla., 1993), and cells of the dentate gyrus migrate from the subgranular layer and into the granular cell layer where they differentiate into hippocampal granule cell neurones (Altman & Das., 1965; Kuhn et al., 1996).

Neurogenesis, albeit to a lesser extent, has also been reported to occur in several other brain areas. For instance, a stem cell population has been reported to reside in the rostral extension of the rostral migratory stream (Gritti et al., 2002), evidence of neurogenesis in the cortex has also been observed (Gould et al., 1999) and bromodeoxyuridine (BrdU) labelling studies have shown the existence of neurogenesis in the Ammon's horn of the hippocampus (Rietze et al., 2000). The medial-rostral part of the substantia nigra pars compacta has also been reported to generate dopamine projection neurones (Zhao et al., 2003), however this remains debatable since multipotent progenitors from this region have only shown to generate mature glial cells in situ (Lie et al., 2002). Furthermore, the failure to show any nigral cells double labelled with BrdU & TH indicates that neurogenesis in the substantia nigra probably does not occur (Frielingsdorf et al., 2004).

The isolation, in vitro proliferation and differentiation of adult neural stem cells has been achieved successfully, with cells from the rodent brain showing the capacity for in vitro neurogenesis when expanded in the presence of epidermal growth factor (EGF) (Reynolds & Weiss., 1992), or basic fibroblast growth factor (bFGF) (Richards et al., 1992; Gritti et al., 1996; Palmer et al., 1995; 1997). Interestingly, while the isolation and culturing of cells from the SVZ have shown self-renewal and multipotent capacities (Gritti et al., 1996), which are characteristics for defining stem cells (Reynolds & Weiss., 1992; 1996; Gage et al., 1995; 2000), there is some debate about the identity of the cells from the dentate gyrus of the hippocampus. Cells of the
hippocampus have been shown to exhibit multipotency and self-renewal properties in vitro (Palmer et al., 1995; 1997), thus suggesting that the cells of the dentate gyrus are stem cells. However, cells dissected specifically from the dentate gyrus show limited self-renewal capacities and unipotency, thus implying that these cells represent a population of restricted progenitors rather than stem cells (Seaberg & van der Kooy, 2002). The multipotency demonstrated by hippocampal cells must therefore arise from another source, and it has been argued that gross dissection of the hippocampus inevitably results in the removal of the adjacent SVZ stem cells, which are the ones responsible for the apparent stem cell properties of the hippocampus (Seaberg & van der Kooy, 2002).

The isolation of adult neural stem cells and their subsequent in vitro differentiation into neurones has now been successfully achieved from a number of non-rodent species including monkeys (Gould et al., 2001), humans (Roy et al., 2000a; 2000b; Arsenijevic et al., 2001) and even from post-mortem human brain tissue (Palmer et al., 2001). Neurones and glia generated from human adult stem cells have also shown appropriate electrophysiological properties thus demonstrating their functionality (Westerlund et al., 2003). While no evidence for functional neurones has been demonstrated following transplantation of adult neural stem cells into the adult brain, several reports clearly indicate that these cells can appropriately respond to environmental signals and differentiate into neural phenotypes following transplantation. For instance, expanded hippocampal progenitors transplanted into the hippocampus of the adult brain have shown to differentiate into neurones when located in the neurogenic region of the dentate gyrus, or into glia when residing outside this area (Gage et al., 1995b). Moreover, transplantation of hippocampus-derived adult progenitors into the rostral migratory stream of rats showed that the majority of grafted cells behave like endogenous progenitors and migrate into the olfactory bulb where they differentiate into neurones (Suhonen et al., 1996).

The ability of adult neural stem cells to generate neural phenotypes both in vitro and in vivo is of particular interest because of their potential clinical use in transplantation therapies for neurodegenerative disease. However, for adult neural stem cells to be a potential source of neurones in for Parkinson's disease, cells must have the capacity to generate not only neurones, but also, dopaminergic neurones. Although the report by
Suhonen et al. showed a small proportion of adult progenitor-derived neurones expressing TH, and therefore possibly being of dopaminergic phenotype, no other evidence for the generation of dopamine neurones has been reported.

In this chapter we assessed the *in vitro* neural differentiation of hippocampal-derived adult neural stem cells following short-term expansion and examined the extent of dopamine differentiation. Since adult neural progenitor cells have previously been shown to respond to environment signals, we also wanted to examine whether these cells could respond to developmental signals and generate midbrain dopamine neurones when injected into the developing ventral mesencephalon of embryonic rats. However, due to inadequate number of cells, transplantation studies could not be undertaken.
5.2 Experimental Procedure

For a detailed description on the protocols used see chapter two, section 2.3.1 & 2.5.1 respectively.

The hippocampi from eight, 6-week-old female Sprague Dawley rats were dissected and enzymatically digested for 45 minutes at 37°C before being dissociated into single cells. Progenitor cells were isolated from the hippocampus using the Percoll buoyancy fraction method (Palmer et al., 1999). Briefly, dissociated hippocampal cells were mixed with 35% Percoll solution and centrifuged for 10 minutes at 1000 x g. Following centrifugation, any white matter and floating debris were removed and the cell pellet was resuspended in 65% Percoll solution and centrifuged again for 10 minutes at 1000 x g. Any remaining floating debris were removed and floating neural progenitors (~2ml above pellet of red blood cells) were collected and washed free of Percoll by centrifugation in DMEM/F12 + 10% fetal calf serum (FCS).

Cells were plated on poly-ornithine and laminin coated Petri dishes at a density of 500,000 cells per dish in DMEM/F12 containing 10% FCS. After 24 hours, media was changed to growth media, which consisting of DMEM/F12, 1% N2 and 20ng/ml bFGF. 75% of growth media was replaced every other day. Initial cultures became confluent within 3-4 weeks at which point they were passaged (see chapter two, section 2.5.3). Cells were then differentiated on chamber slides coated with poly-ornithine and laminin in media consisting of DMEM/F12, 1% N2, 1% FCS & 0.5μg/ml trans-retinoic acid, for 7 days. Following differentiation, cells were fixed in 4% PFA and stained with β-tubulin-III (neuronal marker), GFAP (astrocyte marker) and TH (see chapter two, section 2.6.2).

All experiments were repeated three times and statistical tests of analysis of variance (ANOVA) were analysed using the statistical package Genstat v7.2.
5.3 Results

5.3.1 Proliferation of adult neural progenitor cells

The number of progenitor cells isolated from the hippocampi of 8 adult rats was relatively low and therefore all cells were pooled and plated onto a 100mm culture dish in media containing 20ng/ml bFGF. Unlike the study by Reynolds and Weiss where adult neural progenitor cells were expanded as free-floating aggregates, called neurospheres (Reynolds & Weiss et al., 1992), adult hippocampal progenitors were proliferated as monolayer cultures that adhered to the culture dish as previously described (Palmer et al., 1999). Initial cell cultures became confluent in approximately 3 weeks, and based on their morphological appearance, the cell culture contained a variety of different cell types (Fig. 5.1A). Following passaging, proliferation of the progenitor cells was slower when compared to the cells in the starting culture, as cells were only 75% confluent by 4 weeks in vitro. The morphology of these cells was also distinctly different to those observed in the initial cell culture, with the majority of the cultured cells appearing elongated and phase-bright (Fig. 5.1B).

Although the initial cell culture reached confluence, because of the small size of the cultures, and the progenitor-enrichment passaging protocol, which is used when dissociating adult neural progenitor cells (Palmer et al., 1995), only a small number of viable cells were available for re-plating. In this case, less then 100,000 viable cells were generated following passaging of initial cell cultures. This insufficient number of cells meant that transplantation studies were abandoned since we estimate that at least 250,000 cells per host would be required for effective transplantation.
Fig 5.1 Cell cultures of adult hippocampal neural progenitor cells. Based on their morphological appearance, initial cell cultures contain a variety of different cell types (A). After passaging, the cells are phase bright and elongated and more characteristic of progenitor cells (B). Scale bar = 100μm.

5.3.2 Differentiation of adult neural progenitor cells
To assess whether adult neural progenitor cells had the in vitro capacity to generate neurones, primary (cells that were not proliferated) and expanded progenitor cells were differentiated for 7 days in media containing retinoic acid. Neurones were identified using the neuronal marker β-tubulin-III and astrocytes were identified using the marker GFAP. Figure 5.2 shows the appearance of cells immunopositive for β-tubulin-III and GFAP.

Fig. 5.2 Fluorescent immunochemistry of neurones and astrocytes derived from adult hippocampal progenitor cells. Neurones are stained using β-tubulin (A) and astrocytes are stained using GFAP (B). Cells are visualised using green florescence. All cell bodies are stained blue with Hoechst. Scale bar = 100μm.
The percentage of neurones and astrocytes derived from adult hippocampal neural progenitor cells is shown in figure 5.3.

Both primary and expanded progenitor cells showed the generation of neurones and astrocytes as demonstrated by the expression of the neuronal marker β-tubulin-III and the astrocyte-specific marker glial fibrillary acidic protein (GFAP). The percentage of neurones derived from primary and expanded adult neural progenitor cells was relatively low, with approximately 5% of primary cells and 3% of expanded cells expressing β-tubulin-III (Fig. 5.3). The percentage of astrocytes generated from primary and expanded adult progenitors was much higher than the yield of neurones, with 20-25% of both primary and expanded cells being immunopositive for GFAP (Fig. 5.3). Statistical analysis of the data showed no significant difference in the percentage of neurones and astrocytes generated by primary and expanded progenitors (F(1,59)=2.01, n.s).

To examine the proportion of neurones that were of possible dopaminergic phenotypes, neurones from both primary and expanded cells were stained for tyrosine hydroxylase (TH), which is an enzyme involved in dopamine synthesis. No neurones immunopositive for TH were observed at any time, therefore indicating that neurones were not of dopaminergic phenotypes.

![Figure 5.3 Percentage of neurones and astrocytes derived from adult hippocampal neural progenitor cells. Both primary (P0) and expanded (P1) cell cultures yield relatively low percentages of neurones (black bars), but high yields of astrocytes (white bars). Data expressed as mean ± S.E.M. across three separate experiments.](image)
5.4 Discussion

In addition to the ability of adult neural progenitor cells to generate neurones, the use of these cells in cell replacement strategies for Parkinson’s disease is a particularly attractive alternative to the use of foetal tissue for a number of reasons. Firstly, the use of adult cells is free from ethical issues that are currently surrounding the use of embryonic stem cells. Secondly, adult neural progenitors offer the possibility of autologous transplantation, thus avoiding the issues of rejection which surround the use of foreign tissue, and thirdly, since endogenous adult progenitor cells have shown to generate neurones in response to injury (Arvidsson et al., 2002; Nakatomi et al., 2002) and infusions of growth factors (Kuhn et al., 1997; Pencea et al., 2001 Fallon et al., 2000), the idea of stimulating endogenous stem cells for the induction of self-repair is a real possibility.

Since Parkinson’s disease is primarily characterised by a loss of midbrain dopamine neurones (Samii et al., 2004; Olanow & Tatton, 1999), the ability of progenitor cells to differentiate into neurones of dopaminergic phenotypes is essential. However, while adult neural progenitor cells have shown to generate neurones both in vitro (Gritti et al., 1996; Reynolds and Weiss., 1992; Palmer et al., 1995; 1997; 1999; 2001) and following transplantation into the adult brain (Gage et al., 1995b; Suhonen et al., 1996), the extent of dopaminergic differentiation has not been reported. By isolating adult hippocampal progenitor cells using the Percoll buoyancy faction method (Palmer et al., 1999) and differentiating cells in vitro, we have shown that adult progenitors can generate neurones, which is consistent with previous reports. However, the in vitro generation of neurones is relatively low, and more importantly, adult progenitor cells fail to spontaneously differentiate into dopamine neurones as demonstrated by the lack of neurones being immunopositive for TH.

5.4.1 Proliferation of adult neural progenitor cells

The proliferation of adult neural progenitor cells was successfully achieved following exposure to media containing 20ng/ml bFGF and unlike the neurosphere cultures that have been previously reported (Reynolds & Weiss., 1992), adult progenitor cells were influenced to grow as monolayer cultures by coating the culture dishes with polyornithine and laminin. The initial cell culture contained a variety of different cell types as demonstrated by their distinct morphological characteristics. Although these
cell types were not specifically identified, the initial cell culture has been shown previously to consist of oligodendrocytes, astrocytes, microglia, fibroblasts and immature neurones (Palmer et al., 1995). After passaging, the appearance of cells was markedly different to primary cells. This vast contrast in cell morphologies is probably a result of the absence of glia and fibroblasts from the cell culture, due to the enrichment of progenitor cells during the passaging procedure. It is generally accepted that glia and fibroblasts are more adherent than immature progenitor cells, and therefore, brief treatment with trypsin during passaging can be used to selectively isolate the progenitor cells from the culture. Continued passaging therefore 'filters out' glial cells and in turn enriches the population of adult-derived neural progenitors (Palmer et al., 1995). The removal of glia from the culture following passage could also explain why progenitor-enriched cultures proliferate slower than initial cell cultures. It is possible that glial cells proliferate faster than progenitor cells and therefore, the removal of glial cells during passaging will result in decreased proliferation rates of passaged cultures. Alternatively, since glia are known to aid the maintenance and survival of neurones by secreting trophic factors, their removal may result in the slower proliferation of progenitor cells due to the lack of trophic support.

5.4.2 Adult neural progenitor cells differentiate into neurones and glia in vitro

Consistent with previous reports, which have shown the generation of neural phenotypes from adult neural progenitor cells (Palmer et al., 1995; 1997), the results from this study have also shown that adult hippocampal neural progenitor cells have the capacity to differentiate into cells of neural phenotypes following periods of in vitro expansion. The neuronal yield from both primary and expanded progenitors was however relatively low (~5% and ~3% respectively, Fig. 5.3), whereas the percentage of adult progenitor cell-derived astrocytes was much higher (~25% for primary cells and ~22% for expanded cells, Fig. 5.3). Although the remaining differentiated cells were not identified, a proportion of these cells are likely to be oligodendrocytes as previously shown (Palmer et al., 1995; 1997). It is also probable that some microglia, fibroblasts and undifferentiated progenitor cells remain in the cultures.

The levels of neurogenesis observed in this study are consistent with the results reported by Palmer et al, who have also shown low yields of neurones following differentiation of adult progenitors (Palmer et al., 1999). However, while both studies
have demonstrated low-levels of neurogenesis, there are some differences between the
data reported by Palmer and the results presented here. For instance, Palmer et al
shows a neuronal yield of 1% and 8% for primary and expanded progenitors (which
have also been passaged once), respectively. Our results however, show that primary
cells generate 5% neurones and expanded progenitors generate 3% neurones.
The reasons for these differences are unclear, but perhaps the difference in the
neuronal yield for primary cells may be because of the techniques used for cell
preparation. In the study by Palmer et al., dissected tissue was dissociated into single
cells by filtering cells through a mesh, while we used gentle trituration. Our
preparation technique may have been more favourable and may therefore explain why
more neurones were observed following differentiation of primary cells. In turn, the
higher neuronal yield reported by Palmer following differentiation of expanded
progenitors may have been because of the different differentiation conditions used.
Palmer and colleagues used differentiation media containing 0.1µg/ml of retinoic
acid, while in our study we used a concentration of 0.5µg/ml. Since retinoic acid is a
factor important in promoting the neural differentiation of adult neural progenitor
cells (Takahashi et al., 1999), the difference in retinoic acid concentration may have
contributed to the difference in neurogenesis observed.

Interestingly, it has been reported that adult neural progenitor cells may be a type of
astrocyte since astrocytes have shown to generate neurones both in the subventricular
zone (Doetsch et al., 1999) and in the hippocampus (Seri et al., 2001). Therefore, the
abundant numbers of astrocytes observed in our cultures might reflect either
differentiated astrocytes derived from progenitor cells or a proportion of these adult
progenitor cells. If the astrocytes represent the former, then the multipotent potential
of adult neural progenitor cells is demonstrated. However, if the astrocytes represent
progenitor cells, then this suggests that the in vitro conditions were inadequate for the
differentiation of progenitor cells.
In addition, if the astrocytes are progenitor cells, then the progenitor-enrichment
passaging protocol, which eliminates glia, will reduce the number of progenitors in
the culture, and may explain why adult neural progenitor cells struggle to generate
neurones. In an attempt to increase the number of progenitors in the culture, perhaps
the progenitor-enrichment passaging protocol should be avoided, and all cells should be passaged to ensure that astrocytes are not removed from the culture.

5.4.3 Adult neural progenitor cells fail to generate dopamine neurones

The failure of expanded adult progenitors to differentiate into dopamine neurones was somewhat expected given the low yield of β-tubulin-positive cells. In addition, because embryonic ventral mesencephalic neural progenitor cells fail to generate dopaminergic neurones, or generate extremely low numbers of dopaminergic neurones following expansion, the lack of dopaminergic differentiation following proliferation of adult progenitor cells was not surprising. Because of this detrimental effect of proliferation on the generation of dopamine neurones, we decided to differentiate primary cells (cells that were not proliferated) and assess their potential to generate into dopamine neurones. However, not even primary cells differentiated into dopamine neurones, again not overly surprising given of the low neuronal yield and the inability of expanded progenitors to differentiate into dopaminergic phenotypes.

While evidence for the differentiation of adult neural progenitor cells into mesencephalic dopamine neurones has not been reported, a small proportion of these cells have been shown to differentiate into tyrosine hydroxylase-positive olfactory bulb neurones following transplantation (Suhonen et al., 1996). The lack of in vitro dopamine differentiation is therefore likely to reflect the in vitro conditions, which might be missing essential factors/signals that influence dopaminergic differentiation, rather than an inability of adult neural progenitor cells to differentiate into dopamine neurones. The identity of these factors/signals are unknown, but to try and promote the in vitro dopaminergic differentiation of adult neural progenitor cells, future studies should take known strategies that have been shown to enhance dopaminergic differentiation and apply these conditions to cultures of adult neural progenitors. For instance, the dopaminergic differentiation of adult progenitors may require exposure to a cocktail of different factors, similar to that reported for the dopaminergic differentiation of embryonic neural progenitors (Ling et al., 1998; Carvey et al., 2001).
5.4.4 Adult progenitor cells generate insufficient numbers of cells for transplantation

The isolation of adult neural progenitor cells using the Percoll buoyancy fraction method (Palmer et al., 1999) generates a small number of cells, which means only small-sized cultures can be established. Even when these cultures are confluent, there are still insufficient numbers of progenitor cells available for transplantation. Interestingly, in a recent transplantation study using adult progenitor cells (Dziewczapolski et al., 2003), not only were cells at passage 11 used, but also, cells were isolated using a method other than the Percoll method, probably to generate larger starting cultures. The reason why passage 11 cells were used was not explained, but it suggests that this level of expansion is necessary to generate appropriate numbers of cells. One possible explanation for using such cells may be because the neurogenic capacity of adult neural progenitors has been reported to increase with increased proliferation, possibly because of a result of bFGF-induced recruitment of neurone-competent progenitors (Palmer et al., 1999). However, because Dziewczapolski et al did not mention this in their report, it is unlikely that this was the reason why passage 11 cells were chosen.

The suggestion that neurogenesis increases with proliferation is quite interesting since the opposite has been shown with embryonic rat neural progenitors (see chapter three & Smith et al., 2003). Also, our results do not reflect this finding as we show that primary progenitors generate more neurones than expanded progenitors. The reasons for these conflicting results are unclear, but it is important to note that Palmer et al based their conclusion on the neurogenic potential of primary cells and cultures proliferated for 3, 7, 14 and 28 days. Our conclusion however, was only based on comparing the differentiation of primary cells and cultures proliferated for 21 days. Since initial cell cultures and expanded cultures are distinctly different, direct comparisons between these cell types cannot be made. To confirm our findings we must compare the differentiation of expanded progenitor cultures to cells that have been expanded for different periods of time.

In summary, adult neural progenitor cells can differentiate into neural phenotypes in vitro, however, neurogenesis is low and dopamine differentiation is non-existent. The generation of a large number of cells is also proving difficult, therefore restricting
transplantation studies. Over the past few years research into adult progenitor cells has gone relatively quiet, perhaps because of the problems mentioned above. Substantial progress in this field is therefore required before adult neural stem cells can be considered as a potential source of neurones for transplantation.
Chapter Six

Experiment Four - Evaluation of nigrostriatal lesions in the 129 OLA mouse strain as a suitable model of Parkinson's disease.

Summary

This chapter discusses the use of the 129P2/OLA Hsd mouse strain as a suitable model for Parkinson's disease.

Using the standard medial forebrain bundle (MFB) 6-OHDA lesion, and the induction of rotational behaviour following 5mg/kg amphetamine administration, it was apparent that the resultant rotations were variable and unreliable with no correlation between rotation and dopamine loss. It was therefore necessary to characterise the conditions that gave the optimal rotational response for the 129 OLA mouse strain. Both the unilateral 6-OHDA MFB lesion and the striatal terminal lesion protocols were compared, with mice analysed for appropriate rotational responses induced after the administration of amphetamine and apomorphine at different concentrations. Neither the MFB nor striatal 6-OHDA lesioned mice produced high rotational responses following apomorphine stimulation at any dose. However, mice did respond to amphetamine, with peak rotation dose at 5mg/kg and 10mg/kg for MFB and striatal lesions respectively.

Although bundle lesioned mice responded to amphetamine and apomorphine, the lesion was unreliable with there being no correlation between dopamine loss and rotational asymmetry ($r^2=0.007$ amphetamine, $r^2=0.005$ apomorphine).

Striatal lesions provided more reliable lesions, with stronger correlations between dopamine loss and rotational asymmetry being observed ($r^2=0.70$ amphetamine, $r^2=0.16$ apomorphine).

From this assessment, it seems that the optimal conditions that produce maximum and reliable rotational behaviour recordings for the 129 OLA mouse strain were striatal terminal 6-OHDA lesion followed by rotation measurement after 10mg/kg amphetamine administration.
6.1 Introduction

Parkinson's disease is a progressive neurological disorder that is characterised by a catalogue of movement impairments such as rigidity, tremor and bradykinesia (Olanow & Tatton., 1999; Samii et al., 2004). The disease is primarily caused by the loss of the nigrostriatal dopamine pathway (Olanow & Tatton., 1999), and therefore highlights the importance of striatal dopamine on motor function.

Experimental evidence for the role of dopamine in the striatum, and its effects on motor function first came from dopaminergic stimulation of the rat striatum, which resulted in marked changes in motor response (Ungerstedt et al., 1969). The subsequent pioneering investigations on motor effects following unilateral dopamine depletion using 6-hydroxydopamine (6-OHDA) provided a unique way of analysing dopamine activity by measuring drug-induced motor function (Ungerstedt et al., 1970; Ungerstedt., 1971a; 1971b). Under these conditions rats displayed a turning behaviour referred to as 'rotation', with the degree of rotation being proportional to dopamine loss (Lee et al., 1996) and the direction of rotation being dependent on the rotation-inducing drug used (Ungerstedt., 1971a; 1971b).

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can also be used to lesion the dopamine system because of its specific targeting and destruction of the nigrostriatal dopamine pathway. Its chance discovery following its Parkinsonism-inducing effects in humans (Langston et al., 1983 Ballard et al., 1985), has provided an alternative to the use of 6-OHDA, and has been used to produce motor dysfunctions in a variety of animals including monkeys (Burns et al., 1983; Jenner et al., 1984; Schneider et al., 1987), cats (Schneider & Markham., 1986; Williams & Schneider., 1989) and mice (Heikkila et al., 1984; Ricaurte et al., 1986).

The main advantage of MPTP is that it can be given systematically, unlike 6-OHDA, which requires accurate infusion in the desired brain area. This neurotoxin however causes bilateral dopamine depletion, so animals exhibit profound regulatory deficits in the absence of one normal system to maintain health. Moreover, reliable behavioural tests such as rotation, which are dependant on unilateral asymmetries, cannot be assessed. Furthermore, spontaneous recovery of MPTP-induced deficits has been reported (Iancu et al., 2005 and references therein), which means long-term assessment of behavioural deficits cannot be achieved. In addition, MPTP
neurotoxicity varies in its effects (Giovanni et al., 1994), with some mouse strains being more sensitive than others (Betarbet et al., 2002), and rats being resistant to MPTP toxic insults (Giovanni et al., 1994). It is currently unclear why rats are not susceptible to MPTP, but their inability to breakdown MPTP into the damaging toxic metabolite MPP$^+$ is not the reason since intrastriatal infusion of MPP$^+$ into the rat brain results in insignificant dopamine loss when compared to mice (Giovanni et al., 1994). The temporary deficits caused by MPTP administration, and the variability of its effects, both within and across species has therefore limited the use of MPTP in research.

Although a number of models for Parkinson’s disease exist (see reviews by Betarbet et al., 2002; Shimohama et al., 2003), the 6-OHDA-lesion model of Parkinson’s disease is still well established and is probably the most widely used model in Parkinson’s disease research. This model has without doubt accelerated progress into Parkinson’s disease research by providing a powerful tool to assess the potential of curative treatments by examining the attenuation of 6-OHDA-lesion-induced behavioural deficits. However, while this model has been well established in rats, it is surprising why the 6-OHDA-lesion model of Parkinson’s disease is not more widely used in mice. With the advancements in mouse embryonic stem cell research, the requirement for a mouse 6-OHDA-lesion model of Parkinson’s disease is becoming essential in order to allow the therapeutic potential of mouse embryonic stem cells, and other mouse-derived cell lines to be assessed efficiently without the added complications of immunosuppression.

Although rotational behaviour has been observed in dopamine-depleted mice (Brundin et al., 1986; Barberi et al., 2003; Iancu et al., 2005), the resultant rotational bias and the methods used to induce rotational behaviour in these studies vary considerably, with differences in lesion type, drug, and drug doses used. For instance, the unilateral, 6-OHDA intrastriatal lesions used by Brundin et al., (1986) produced low rotational asymmetry following 2.5mg/kg amphetamine stimulation. In contrast, much better rotations were reported by Barberi et al., (2003) following striatal lesions and stimulation by 10mg/kg amphetamine and apomorphine. However, while good rotations were observed by Barberi and colleagues, the drug doses used seem
excessive, particularly with respect to apomorphine. This high-dose apomorphine-induced rotation suggests that no supersensitivity of post-synaptic dopamine receptors occurred, even though increased receptor binding has been previously reported following striatal 6-OHDA lesions in mice (Bensadoun et al., 2000). In addition, while MFB lesions used by Iancu et al., (2005) have been shown to produce good rotational behaviour following 2.5mg/kg amphetamine challenge, lesions were inconsistent and are therefore unreliable.

It is clear from these studies that a standard protocol for developing good rotational bias following the unilateral 6-OHDA lesions in mice has yet to be established. This chapter aims to evaluate the 129 OLA mouse strain for its suitability to produce rotational behaviour following 6-OHDA lesioning, and to establish the conditions that are necessary to produce optimal rotational bias that reflect dopamine depletion. Both medial forebrain bundle (MFB) and striatal terminal 6-OHDA lesions have been compared for rotation after amphetamine and apomorphine stimulation. Drug dose giving peak rotational behaviour has also been calculated for amphetamine and apomorphine.

This chapter provides a comprehensive, detailed assessment of lesion type, drug and drug dose required to produce a standard, reproducible model where rotational bias reflects dopamine loss in the 129 OLA mouse strain.
6.2 Experimental Procedure

A full, detailed description of the experimental procedures can be found in chapter two, section 2.7.1 & 2.8.2 and will only be briefly described here.

Prior to commencing behavioural testing, all mice underwent ten minutes of habituation to their testing environment. This eliminated any novel environmental stimuli that might have interfered with rotational recordings.

When testing amphetamine-induced rotations, d-amphetamine was used rather than the more potent methamphetamine. Behavioural testing lasted 90 minutes, with each mouse having its rotational behaviour recorded for one minute at each interval, for a total of fifteen intervals, with each interval lasting six minutes.

Due to the faster metabolism and clearance of apomorphine compared to amphetamine, apomorphine-induced rotations lasted for 48 minutes with each mouse having its rotational behaviour recorded for one minute at each interval, for a total of eight intervals, with each interval lasting six minutes.

Both amphetamine and apomorphine were dissolved in 0.01% ascorbic saline to prevent the oxidation of the drugs, and were administered via intraperitoneal and subcutaneous injection respectively. Mice were given at least 7 days to recover between each amphetamine dose and at least 4 days between each apomorphine dose, to ensure that drugs had been eliminated from the system.

Stimulation of lesioned mice by amphetamine results in rotations ipsilateral to the side of the lesion. Apomorphine stimulation of the lesioned mice results in rotations contralateral to the side of the lesion. For this reason, when plotting rotational data, ipsilateral rotations were given positive values whereas contralateral rotations were given negative values, thus reflecting the direction of rotation.

Statistical tests of analysis of variance (ANOVA) were analysed using the statistical package Genstat v7.2, with further analysis of the results being achieved by using the Neuman Keuls t-test.
6.3 Results

6.3.1 Effect of ascorbic acid on amphetamine-induced rotations

Mice (n=5) with 6-OHDA, medial forebrain bundle lesions were tested for rotational bias following stimulation with 2.5mg/kg amphetamine, supplemented with or without 0.01% ascorbic acid. Mice were also observed for rotational asymmetry following 0.01% ascorbic saline injection (0mg/kg – control group).

The addition of ascorbic acid to amphetamine had no effect on rotational behaviour, with both amphetamine groups (with and without ascorbic acid) rotating an average of 3.3 times per minute. As expected, the control group displayed little rotational behaviour (Fig. 6.1).

Statistical analysis of data confirmed these observations. Although a significant difference in rotations across groups was shown (F(2,22)=111.21, p<0.001), further analysis showed no significant difference between the amphetamine groups (NK: t(2,2)=0.223, n.s). However, 2.5mg/kg amphetamine-induced rotations (with and without ascorbic acid) showed significantly higher rotational bias compared to the control group (NK: t(3,2)=18.436, p<0.01 amphetamine) & (NK: t(2,2)=18.212, p<0.01 amphetamine + ascorbic acid).

Fig. 6.1 Effect of ascorbic acid on rotational behaviour following 2.5mg/kg amphetamine stimulation. Statistical analysis shows no effect of ascorbic acid on rotational behaviour.
6.3.2 Amphetamine-induced rotations on medial forebrain bundle (MFB) 6-OHDA lesioned mice

24 mice were tested for their rotational behaviour over time after the administration of amphetamine at 0mg/kg (0.01% ascorbic saline injection) 2.5mg/kg, 5mg/kg and 10mg/kg. Mice injected with 0mg/kg amphetamine showed little rotational behaviour. At 2.5mg/kg, mice showed good rotation, with peak rotation (6 rotations/minute) at interval 3 (18 minutes) which stabilised over the following three intervals. After the sixth interval (36 minutes), rotational behaviour decreased to 1.71 rotations/minute (Fig. 6.2).

Rotations at 5mg/kg were similar to the rotational behaviour observed at 2.5mg/kg with peak rotation at around 6 rotations/minute. However, rotation was more stable at this dose with a reduction in drug-induced response occurring after interval ten (60 minutes), compared to interval six (36 minutes) at a dose of 2.5mg/kg (Fig. 6.2).

At a dose of 10mg/kg, the induced rotational behaviour was notably lower than the rotation induced at both 2.5mg/kg and 5mg/kg. After the sixth interval, there was an increase in rotational response that remained stable until a small drop at the final two intervals (Fig. 6.2).

Results show a significant effect of amphetamine dose on rotational response ($F_{(3,1380)}=40.31$, $p<0.001$), with further analysis indicating that 5mg/kg amphetamine induces rotational responses that were significantly higher than rotations produced by 10mg/kg and 0mg/kg amphetamine (NK: $t_{(3,3)}=13.85$, $p<0.01$) & (NK: $t_{(4,3)}=8.624$, $p<0.01$) respectively, but not than 2.5mg/kg (NK: $t_{(2,3)}=1.957$, n.s). Rotation induced by 2.5mg/kg was significantly higher than the rotation produced from both 10mg/kg and 0mg/kg (NK: $t_{(2,3)}=6.667$, $p<0.05$) & (NK: $t_{(3,3)}=11.896$, $p<0.01$) respectively. Rotations induced by 10mg/kg were also significantly higher than 0mg/kg (NK: $t_{(2,3)}=5.229$, $p<0.05$) (Fig. 6.2).
Fig. 6.2 Amphetamine induced rotations at different sample intervals on bundle lesioned mice. Amphetamine dose has a significant effect on the induction of rotational response ($p<0.001$).

6.3.3 Amphetamine-induced rotations on striatal 6-OHDA lesioned mice

7 mice were tested for their rotational behaviour over time after the administration of amphetamine at 0mg/kg (0.01% ascorbic saline injection) 2.5mg/kg, 5mg/kg, 10mg/kg and 15mg/kg.

Mice injected with 0mg/kg amphetamine showed little rotational behaviour (Fig. 6.3). At 2.5mg/kg, mice showed good rotations during the early intervals with rotations reaching over 6 rotations/minute. However, after interval 5 (30 minutes) the effects on rotation rapidly decreased with rotation at the final interval being 1.43 rotations/minute (Fig. 6.3). At 5mg/kg, rotational behaviour was, in general, higher than that produced from 2.5mg/kg. By interval 6 (30 minutes), rotation had reached its peak (over 8 rotations/minute) with rotation stabilising over the next few intervals.

After 72 minutes (interval 12) rotation rate decreases sharply ending at 3.57 rotations/minute at the final interval (Fig. 6.3). 10mg/kg amphetamine produced a high rotation rate with rotations of 9.71 per minute by the third interval (18 minutes). Rotation rate was stable for a long period of time, reaching values of 11.29 rotations/minute. After the thirteenth interval (78 minutes) rotation rate reduced but
was still relatively high with rotations of 8.14 rotations/minute at the final interval (Fig. 6.3). At 15mg/kg, there was a steady increase in rotational behaviour with time, with a peak rotation rate of 8.29 rotations/minute occurring in the final interval (Fig. 6.3).

Analysis of variance (ANOVA) indicated a significant effect of amphetamine dose on rotational response ($F_{(4,450)}=180.07$, $p<0.001$). Further analysis shows that 10mg/kg amphetamine-induced rotation was significantly higher than rotations produced by 15mg/kg, 5mg/kg, 2.5mg/kg and 0mg/kg amphetamine (NK: $t_{(3,4)}=16.68$, $p<0.01$), (NK: $t_{(4,4)}=14.324$, $p<0.01$), (NK: $t_{(2,4)}=14.324$, $p<0.01$) & (NK: $t_{(5,4)}=36.865$, $p<0.01$) respectively. 5mg/kg amphetamine-induced rotations were significantly higher than rotations induced by 2.5mg/kg (NK: $t_{(3,4)}=8.533$, $p<0.01$) and 0mg/kg (NK: $t_{(4,4)}=22.54$, $p<0.01$) but not significantly different than the rotations induced by 15mg/kg amphetamine (NK: $t_{(2,4)}=2.355$, n.s). 15mg/kg amphetamine rotations were significantly higher than the rotations induced by 2.5mg/kg amphetamine (NK: $t_{(2,4)}=6.178$, $p<0.05$), with rotations induced by 2.5mg/kg being significantly higher than behavioural rotation at 0mg/kg (NK: $t_{(2,4)}=14.008$, $p<0.01$) (Fig. 6.3).

![Fig. 6.3 Amphetamine induced rotations at different sample intervals on striatal lesioned mice. Amphetamine dose has a significant effect on the induction of rotational response (p<0.001).](image-url)
6.3.4 Amphetamine-induced rotations on non-lesioned (control) mice

Non-lesioned mice were tested for amphetamine-induced rotation at all doses (0mg/kg-15mg/kg). This was necessary to confirm that rotations observed in the mice were due to the lesioning of the dopamine system and not any other factor (Fig. 6.4). Following amphetamine simulation, the activity of mice was amplified and slight rotational behaviour was seen, this rotation was however insignificant at all doses ($F_{(4,525)}=1.88$, n.s).

The rotational response after amphetamine stimulation on control mice across time can be seen in Fig. 6.4 with Fig. 6.5 showing individual dose-response graphs.

![Graph showing amphetamine induced rotations at different sample intervals on non-lesiond (control) mice. Statistical analysis shows that there is no significant effect of amphetamine on the rotational behaviour of non-lesioned (control) mice at any dose.](image-url)

*Fig. 6.4* Amphetamine induced rotations at different sample intervals on non-lesioned (control) mice. Statistical analysis shows that there is no significant effect of amphetamine on the rotational behaviour of non-lesioned (control) mice at any dose.
Fig. 6.5 Individual dose-response graphs for amphetamine-induced rotations on non-lesioned (control) mice at 2.5mg/kg (a), 5mg/kg (b), 10mg/kg (c) & 15mg/kg (d).
6.3.5 *Apomorphine-induced rotations on medial forebrain bundle 6-OHDA lesioned mice*

As well as mice being tested for their rotational behaviour after the administration of amphetamine, rotation was also assessed after apomorphine stimulation. 8 mice with unilateral MFB 6-OHDA lesions received doses of apomorphine at 0mg/kg (0.01% ascorbic saline injection), 0.01mg/kg, 0.05mg/kg, 2.5mg/kg, 5mg/kg and 10mg/kg, with rotation lasting 48 minutes (8 intervals).

Mice injected with 0mg/kg, 0.01mg/kg and 0.05mg/kg showed little rotational response across the whole eight intervals (average rotations of 0.19, 0.08 and 0.05 rotations/minute respectively, Fig. 6.6). Doses of 2.5mg/kg produced better rotation compared to 0mg/kg, 0.01mg/kg and 0.05 mg/kg, with an average rotation rate of 2.76 rotations/minute. Rotation rate was stable for a short period, with rotation at intervals 2-4 (12-24 minutes) being around 3 rotations/minute. Over time, rotation slowly decreased, with rotation at the final interval being 2.12 rotations/min (Fig. 6.6).

At a dose of 5mg/kg, rotation was more stable for a longer period of time, with rotation rates of around 3 rotations/minute being established and maintained from the second interval (12 minutes) until the final interval (Fig. 6.6). At 10mg/kg apomorphine produced rotations that were stable throughout the 48 minute testing session but with rotations being relatively low (less than 2 rotations/minute) compared to the rotations produced by 2.5mg/kg & 5mg/kg apomorphine (Fig. 6.6).

Analysis of variance (ANOVA) indicated a significant effect of apomorphine dose on rotational response (*F*(5,336)=31.43, *p*<0.001).

Further analysis showed that rotations at 5mg/kg apomorphine were significantly higher than rotations produced by 10mg/kg, 0.05mg/kg, 0.01mg/kg and 0mg/kg apomorphine (NK: *t*(3,5)=6.519, *p*<0.05), (NK: *t*(6,5)=11.704, *p*<0.01), (NK: *t*(5,5)=11.593, *p*<0.01) & (NK: *t*(4,5)=11.185, *p*<0.01) respectively, but not than 2.5mg/kg (NK: *t*(2,5)=1.667, n.s). 2.5mg/kg apomorphine-induced rotations were significantly higher than rotations induced by 10mg/kg (NK: *t*(2,5)=4.852, *p*<0.05), 0.05mg/kg (NK: *t*(3,5)=10.037, *p*<0.01), 0.01mg/kg (NK: *t*(4,5)=9.926, *p*<0.01) and 0mg/kg (NK: *t*(3,5)=9.519, *p*<0.01).

Apomorphine doses of 0.05mg/kg, 0.01mg/kg and 0mg/kg did not produce rotations that were significantly different to each other (NK: *t*(2,5)=0.407, n.s), (NK: *t*(3,5)=0.519, n.s) & (NK: *t*(4,5)=0.111, n.s) respectively (Fig. 6.6).
Fig. 6.6 Apomorphine-induced rotations at different sample intervals on medial forebrain bundle lesioned mice. Statistical analysis shows that there is a significant effect of apomorphine dose on the rotational behaviour of lesioned mice (p<0.001). Negative values = contralateral rotations.

6.3.6 Apomorphine-induced rotations on striatal 6-OHDA lesioned mice

7 mice with unilateral striatal 6-OHDA lesions, received subcutaneous injections of apomorphine at doses of 15mg/kg, 10mg/kg, 5mg/kg, 2.5mg/kg, 0.05mg/kg and 0mg/kg (0.01%ascorbic saline injection).

Neither 0mg/kg nor 0.05mg/kg apomorphine produced any behavioural rotation. There was little rotational behaviour with 2.5mg/kg apomorphine, with rotations fluctuating at 1 rotations/minutes or below across the whole 8 intervals, with the average rotation being 0.588 rotations/minute (Fig. 6.7).

Rotation after 5mg/kg apomorphine administration produced a small rotational response that reduced slowly over the second to fifth interval (12-30 minutes) before stabilising. Peak rotation occurred at interval two (1.29 rotations/minute) but by the final interval rotation had reduced to 0.57 rotations/minutes. The average rotation over the entire eight intervals (48 minutes) was 0.69 rotations/minute (Fig. 6.7).
Rotations at 10mg/kg apomorphine produced the best rotations of the group with the first four intervals having rotations of nearly 3 rotations/minute. After the fourth interval, rotation decreased sharply with rotation falling below 1 rotations/minute after the fifth interval (30 minutes). Average rotation over the entire eight intervals was 1.89 rotations/minute (Fig. 6.7).

15mg/kg apomorphine-induced rotations were relatively low, with no rotations above 1 rotations/minute after the first interval. The average rotation over the entire eight intervals was 0.73 rotations/minute (Fig. 6.7).

Analysis of variance (ANOVA) indicated a significant effect of apomorphine dose on rotational response ($F_{(5,288)}=36.21$, $p<0.001$).

Further analysis of results showed that 10mg/kg apomorphine produced rotations that were significantly higher than the rotations produced at 15mg/kg, 5mg/kg, 2.5mg/kg, 0.05mg/kg and 0mg/kg (NK: $t_{(2,5)}=6.902$, $p<0.01$), (NK: $t_{(3,5)}=8.754$, $p<0.01$), (NK: $t_{(4,5)}=11.111$, $p<0.01$), (NK: $t_{(5,5)}=13.847$, $p<0.01$) and (NK: $t_{(6,5)}=15.001$, $p<0.01$) respectively. Doses of 15mg/kg apomorphine produced rotations that were significantly higher than the rotations produced at 0.05mg/kg (NK: $t_{(4,5)}=6.944$, $p<0.05$), and 0mg/kg (NK: $t_{(5,5)}=8.098$, $p<0.05$), but not significantly different to 5mg/kg (NK: $t_{(2,5)}=1.852$, n.s) and 2.5mg/kg (NK: $t_{(3,5)}=4.209$, n.s).

5mg/kg also produced rotations that were significantly higher than the rotations after 0.05mg/kg and 0mg/kg apomorphine administration (NK: $t_{(3,5)}=5.093$, $p<0.05$) & (NK: $t_{(4,5)}=6.247$, $p<0.05$) respectively. This dose however, did not produce rotations that were significantly different to the rotations corresponding to dose 2.5mg/kg (NK: $t_{(2,5)}=2.357$, n.s).

Rotations at 2.5mg/kg, 0.05g/kg and 0mg/kg apomorphine were not significantly different to each other (Fig. 6.7).
Fig. 6.7 Apomorphine-induced rotations at different sample intervals on striatal lesioned mice. Statistical analysis shows that there is a significant effect of apomorphine dose on the rotational behaviour of lesioned mice (p<0.001). Negative values = contralateral rotations.

6.3.7 Apomorphine-induced rotations on non-lesioned (control) mice
Non-lesioned mice were tested for apomorphine-induced rotation at all doses (0mg/kg-15mg/kg). This was necessary to confirm that rotations observed in the mice were due to the lesioning of the dopamine system and not any other factor (Fig. 6.8). There was no significant rotational behaviour at any dose. The rotational response after apomorphine stimulation on control mice across time can be seen in Fig. 6.8 with Fig. 6.9 showing individual dose-response graphs.
Fig. 6.8 Apomorphine-induced rotations at different sample intervals on non-lesioned (control) mice. Statistical analysis shows that there is no significant effect of apomorphine on the rotational behaviour of non-lesioned (control) mice at any dose.
Fig. 6.9 Individual dose-response graphs for apomorphine-induced rotations on non-lesioned (control) mice at 0.01 mg/kg (a), 0.05 mg/kg (b), 2.5mg/kg (c), 5mg/kg (d), 10mg/kg (e) & 15mg/kg (f).
6.3.8 Comparison of MFB and striatal 6-OHDA lesioned mice

6.3.8.1 Dose-response curves of amphetamine and apomorphine

The average rotational response for MFB and striatal lesioned mice at each dose of amphetamine and apomorphine is shown in Fig. 6.10.

Mice with MFB lesions show dose-dependent rotational behaviour after the stimulation by either amphetamine \( (F(3,95) = 3.20, p<0.05) \) or apomorphine \( (F(5,42) = 5.13, p<0.001) \). At 0mg/kg amphetamine (control), rotation is negligible. Doses of 2.5mg/kg and 5mg/kg amphetamine induce rotations that are higher than rotations at 0mg/kg, with mean rotations reaching at least 4 rotations/minute. There is a decrease of rotational behaviour at 10mg/kg amphetamine where mean rotation decreases to less than 2 rotations/minute. Doses of 0mg/kg and 0.05mg/kg apomorphine result in little or no rotation, whereas mean rotations of 2.78 and 3.18 rotations/minutes are observed at doses of 2.5mg/kg and 5mg/kg apomorphine respectively. At 10mg/kg there is a reduction in mean rotational response to 1.47 rotations/minute (Fig. 6.10a).

Striatal 6-OHDA lesioned mice also show a significant dose-dependent effect following either amphetamine \( (F(4,30) = 22.30, p<0.001) \) or apomorphine challenge \( (F(5,36) = 11.10, p<0.001) \). Amphetamine dose of 10mg/kg produced peak rotation of 9.69 rotations/minute, with doses of 2.5mg/kg, 5mg/kg and 15mg/kg giving rotations of 3.9, 6 and 5.39 rotations/minute respectively. There was little rotation at 0mg/kg (control) with average rotations of 0.3 per minute. Closer analysis of the results show the rotational response at 10mg/kg being significantly higher than at 5mg/kg, 15mg/kg, 2.5mg/kg & 0mg/kg \( (NK: t(2,4) = 5.118, p<0.05) \), \( (NK: t(3,4) = 5.964, p<0.05) \), \( (NK: t(4,4) = 8.031, p<0.05) \) & \( (NK: t(5,4) = 13.024, p<0.01) \) respectively.

Doses of 5mg/kg, 15mg/kg and 2.5mg/kg produced rotations that were not significantly different from each other, but were significantly different when compared to 0mg/kg \( (NK: t(4,4) = 7.906, p<0.05) \), \( (NK: t(3,4) = 7.060, p<0.05) \) & \( (NK: t(2,4) = 4.993, p<0.05) \). Apomorphine-induced rotations on striatal lesioned mice produced relatively low rotation rates. All doses induced rotations of less than 2 rotations/minute, with 10mg/kg apomorphine producing peak rotation of 1.74 rotations/minute. Analysis of the results show that the rotations induced after 10mg/kg apomorphine administration were significantly higher than the rotations at all other doses \( (NK: t(2,5) = 4.998, p<0.05) \), \( (NK: t(3,5) = 5.047, p<0.05) \), \( (NK: t(4,5) = 5.344, p<0.05) \).
& (NK: \( t_{(5,5)} = 7.917, P<0.05 \)) for doses 15mg/kg, 5mg/kg, 2.5mg/kg, 0mg/kg & 0.05mg/kg respectively.

Rotations induced by 15mg/kg, 5mg/kg, 2.5mg/kg, 0.05mg/kg & 0mg/kg apomorphine were not significantly different from each other (Fig. 6.10b).

**Fig. 6.10** Dose-response curves for amphetamine and apomorphine-induced rotations show the average rotational response at each drug dose for MFB (a) and Striatal (b) lesioned mice. Positive values indicate ipsilateral rotations and negative values indicate contralateral rotations.
6.3.8.2 Comparison of amphetamine and apomorphine-induced rotations

Although amphetamine stimulation in the MFB lesion model induces slightly higher rotations than those produced by apomorphine (Fig. 6.11a), statistical analysis shows no significant difference in the rotational behaviour induced following amphetamine and apomorphine stimulation at 2.5mg/kg, 5mg/kg and 10mg/kg (NK: \( t(2,3)=1.353, \) n.s), (NK: \( t(2,3)=1.623, \) n.s) & (NK: \( t(2,3)=0.487, \) n.s) respectively (Fig. 6.11a). The striatal lesion model however, shows a significant difference in drug effect with amphetamine proving a better pharmacological stimulant in inducing rotational bias at all doses except for 0mg/kg (p<0.01) (Fig. 6.11b). Interestingly peak effects of both amphetamine and apomorphine are different for the two lesion models, with peak dose of amphetamine and apomorphine occurring at 5mg/kg for the MFB lesion group and at 10mg/kg for the striatal lesion group (Fig. 6.11a & b).

Fig. 6.11 Comparison of amphetamine and apomorphine-induced rotations on 6-OHDA lesioned mice. Effects of amphetamine and apomorphine are similar on MFB lesioned mice (a), however amphetamine was more successful at inducing rotations in the striatal lesion group (b). Peak drug effects occurred at 5mg/kg for both drugs in the MFB lesion group compared to 10mg/kg for both drugs in the striatal lesion group. **p<0.01
6.3.9 **Bundle vs. Striatal lesions**

6.3.9.1 **Amphetamine-induced rotations**

Fig 6.12 and 6.13 show the comparison between the effects of amphetamine-induced rotations at different doses on both striatal and medial forebrain bundle lesioned mice. At a dose of 2.5 mg/kg amphetamine, the rotational behaviour for both lesion models is very similar (Fig. 6.12a). There is no significant difference between lesion groups at this dose (NK: \( t_{(2,3)} = 0.214, \text{n.s} \)), with the average rotation rate at this dose for striatal and bundle lesioned mice being 3.77 and 4.13 rotations/minute respectively.

At 5 mg/kg amphetamine, the rotational behaviour for both lesion models are also similar to each another (Fig. 6.12b), with no significant difference of rotation between the two (NK: \( t_{(2,3)} = 1.189, \text{n.s} \)).

10 mg/kg amphetamine reveals a large rotational difference between the two groups (Fig. 6.12c), with striatal lesions rotating significantly higher than bundle lesioned animals (NK: \( t_{(2,3)} = 7.544, p<0.05 \)).

Statistical analysis of the results confirm a difference in rotations after amphetamine stimulation on different lesion groups (\( F_{(3,122)} = 3.04, p<0.05 \)).
Fig. 6.12 There is a significant difference of lesion group after amphetamine-induced rotations (p<0.05), with 10mg/kg amphetamine being more effective on striatal than bundle lesioned (p<0.05).
Fig. 6.13 Effect of amphetamine on lesion groups is similar at doses of 2.5mg/kg and 5mg/kg. At 10mg/kg, amphetamine is more effective at producing rotations on striatal lesion models than bundle lesions. *p<0.05.

6.3.9.2 Apomorphine-induced rotations

Fig 6.14 and 6.15 show the comparison between the effects of apomorphine-induced rotations at different doses on both striatal and medial forebrain bundle lesioned mice. At a dose of 0.05mg/kg apomorphine, there is negligible rotation of mice with both bundle and striatal lesions, with rotations averaging -0.04 and 0.01 rotations/minute respectively (Fig. 6.14a).

At 2.5mg/kg and 5mg/kg, apomorphine-induced rotations appear to be more effective on the bundle lesioned animals compared to the striatal lesioned mice, with rotations of -2.78 and -3.18 compared to -0.66 and -0.72 rotations/minute (Fig. 6.14 b & c). Statistical analysis of these results indicate a significant differences in rotation (NK: \( t_{(2,4)} = 4.198, p<0.05 \)) & (NK: \( t_{(2,4)} = 4.871, p<0.05 \)).

10mg/kg apomorphine rotations are similar, with average rotations of -1.47 rotations/minute for bundle lesion animals, and -1.78 rotations/minute for striatal lesioned mice (Fig 6.14d). There is no significant difference between these two groups at this dose (NK: \( t_{(2,4)} = 0.614, \text{n.s} \)).
The difference in rotational behaviour after apomorphine stimulation on the two lesioned groups is confirmed by statistical analysis of variance ($F(4,78)=3.24$, $p<0.05$).

**Fig. 6.14** There is a significant difference of lesion group after apomorphine-induced rotations ($p<0.05$). Bundle lesion group at doses of 2.5mg/kg & 5mg/kg induce significantly higher rotations compared to striatal lesion groups ($p<0.05$).
Fig. 6.15 Effects of apomorphine on lesion groups are similar at 10mg/kg but not at 2.5mg/kg and 5mg/kg where bundle lesions are more responsive. *p<0.05.

6.3.9.3 Comparison of dose-response

The dose-response curves in Fig. 6.10 for bundle and striatal lesioned mice have been superimposed for direct comparison, as shown in Fig. 6.16a.

It appears that the striatal lesioned animals require higher doses of amphetamine and apomorphine to produce peak rotations compared to bundle lesion animals. For example, with bundle lesions 5mg/kg amphetamine and apomorphine induce the peak rotational response. However in striatal lesioned mice, peak rotation occurs at 10mg/kg for both amphetamine and apomorphine.

Another observation is that striatal lesioned animals respond better to amphetamine with greater rotations rates compared to bundle lesions (Fig. 6.16b).

Although apomorphine produces relatively low rotations, it is more effective on bundle lesions than on striatal lesions, with doses of 2.5mg/kg and 5mg/kg inducing the best rotations (Fig. 6.16c)

With the aid of statistical regression, it is possible to calculate statistically the doses of amphetamine and apomorphine that give peak rotations for both bundle and striatal
lesioned animals (Fig. 6.17). Regression analysis on amphetamine-induced rotations on bundle and striatal lesioned animals calculated peak rotations of 5.1 and 8.8 per minute at doses of 5.46mg/kg and 9.43 mg/kg amphetamine respectively (Fig. 6.17a). Regression analysis on apomorphine-induced rotations on bundle and striatal lesioned animals calculated peak rotations of 3.5 and 1.4 per minute at doses of 5.66mg/kg and 9.25 mg/kg apomorphine respectively (Fig. 6.17b).

**Fig. 6.16** Dose-response curves of amphetamine and apomorphine for both lesion groups (a). Dose-response curves for amphetamine (b) and apomorphine (c) are also shown separately for both lesion groups.
Fig. 6.17 Regression analysis of dose-response curves for amphetamine (a) and apomorphine (b) for both lesion groups.
6.3.10 Rotation vs. dopamine cell loss

The extent of dopamine cell loss was analysed in both lesion models (Fig. 6.18) by counting and comparing the number of TH positive cells in the substantia nigra, on both the lesioned and non-lesioned side of the brain (Table 6.1). Counts were obtained from at least three 60μm brain sections, and the average percentage dopamine cell loss was calculated. The mean cell loss for MFB and striatal 6-OHDA lesions were 68.5% and 69.3% respectively, however MFB lesions were significantly more variable than the striatal lesions \( F(2,5) = 63.66, p<0.001 \).

Fig 6.19 shows the correlation between dopamine cell loss and peak drug-induced rotations for each animal with either a MFB 6-OHDA lesion or a striatal 6-OHDA lesion. Medial forebrain bundle lesioned animals show sporadic results with no pattern between dopamine cell loss and rotation following amphetamine and apomorphine-induced rotations. Linear regression analysis for MFB lesioned animals following amphetamine and apomorphine-induced rotations are \( r^2 = 0.007 \) & \( r^2 = 0.005 \) respectively. Striatal lesions however, show stronger correlations between dopamine cell loss and rotation following amphetamine and apomorphine stimulation with \( r^2 \) values of 0.70 and 0.16 respectively.

**Fig. 6.18** TH immunohistochemical staining in brain sections moving rostral-caudal from left to right through the levels of the striatum and the substantia nigra. MFB lesions show clear depletion of TH staining on right hand side, in both the striatum and the substantia nigra (A). Striatal lesions show partial depletion of TH staining on right hand side in the striatum and the substantia nigra (B). Scale bar = 1.5mm
Table 6.1. Comparison of the percentage dopamine cell loss and peak drug-induced rotation scores for MFB and striatal lesioned animals.

**MFB Lesions**

<table>
<thead>
<tr>
<th>Animal number</th>
<th>% Dopamine neurone loss</th>
<th>Rotation score</th>
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</thead>
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<tr>
<td></td>
<td></td>
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</tr>
<tr>
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<td>77.0</td>
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</tr>
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<td>22</td>
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**Striatal Lesions**

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<th>Animal number</th>
<th>% Dopamine neurone loss</th>
<th>Rotation score</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>10.5</td>
</tr>
</tbody>
</table>

* 2 mice with MFB lesions and 1 mouse with a striatal lesion died before the time of histological examination. Therefore table only shows the results from 22 MFB lesioned and 6 striatal lesioned animals.
Fig. 6.19 Comparison of dopamine cell loss and rotation for each animal. Linear regression indicated that there was a stronger correlation between dopamine cell loss and rotations with the Striatal lesion group compared to MFB lesion group after amphetamine and apomorphine stimulation ($r^2 = 0.70$ & $0.16$ vs. $0.007$ & $0.005$) respectively.
6.4 Discussion

The data reported in this chapter show that unilateral 6-OHDA lesions of either the medial forebrain bundle or the striatum produce marked and significant rotational bias following pharmacological stimulation. The frequency of rotational asymmetry however, showed substantial variability with lesion type, drug, and drug dose used.

Rotational behaviour was initiated following the administration of the stimulants amphetamine and apomorphine. Amphetamine is an indirect agonist causing the release of dopamine from dopamine terminals (Ungerstedt., 1971a; Kelly et al., 1975), whereas apomorphine is a direct agonist, and stimulates post-synaptic dopamine receptors (Ungerstedt., 1971b; Kelly et al., 1975).

Unilateral destruction of the nigrostriatal dopamine pathway results in the removal/loss of pre-synaptic dopamine terminals, decreased innervation of post-synaptic striatal neurones and up-regulation and hypersensitivity of post-synaptic dopamine receptors (compensation for the loss of dopamine input). Consequently the two drugs have opposing effects on the direction of rotational bias.

Amphetamine causes greater dopamine release and striatal stimulation on the intact side compared to the lesioned side (due to the loss of dopamine neurones on the lesion side), an imbalance that results in marked motor impairments and net ipsilateral rotations (towards the lesioned side) (Ungerstedt., 1971a). In contrast, apomorphine produces contralateral rotations (away from the lesioned side) due to higher dopaminergic excitation of the lesioned striatum because of the up-regulation and hypersensitivity of post-synaptic dopamine receptors (Ungerstedt., 1971b).

In addition to the different mechanisms of actions of the two drugs, amphetamine and apomorphine also differ in their stability, with apomorphine being rapidly oxidised into an inactive form. Supplementing apomorphine with the antioxidant, ascorbic acid prolongs apomorphine activity, however, apomorphine is still cleared and metabolised relatively quickly compared to amphetamine, hence reflecting the difference in rotation trial times.

Since ascorbic acid had no influence on the rotational behaviour induced in dopamine-depleted mice, it was decided that amphetamine as well as apomorphine would be supplemented with 0.01% ascorbic acid to standardise all conditions.
6.4.1 Drug-induced rotations

A short period of time was taken for the effects of the drugs to induce rotations, but once established a clear pattern emerged with amphetamine and apomorphine-induced rotations declining with time, presumably because of metabolism of the drugs. However, at high doses of amphetamine (10mg/kg and 15mg/kg for bundle and striatal lesions respectively), the reverse was observed, with rotations increasing with time. The reason for this increased rotational activity is unclear, but perhaps these high doses of amphetamine stimulated not only the denervated dorsal striatum, but also, other intact dopaminergic regions, including the ventral striatum. Since this striatal region is involved in the initiation of stereotypical behaviour, such as repetitive limb and head movements, sniffing, and oro-facial stereotypies (Iancu et al., 2005), and because stereotypy is dopamine dependent (Creese & Iversen, 1973), it is likely that stereotypy would have been induced at these high amphetamine doses. Such stereotypic behaviour would have decreased rotational bias, in a similar fashion to the way that high-dose amphetamine-induced stereotypy has been reported to decrease locomotor activity in non-lesioned rats (Gentry et al., 2004). As time elapsed and amphetamine is metabolised, stereotypy would decrease and rotational behaviour would be enhanced, thus explaining why rotational behaviour at high drug doses is delayed and increases with time.

The possible reasons why this phenomenon occur at different doses for the different lesion groups in discussed in section 6.4.3.

6.4.2 Amphetamine and apomorphine stimulation of non-lesioned (control) animals

Amphetamine and apomorphine stimulation of non-lesioned mice was necessary to confirm that the drug-induced rotations seen in lesioned animals were due to the depletion of the dopamine pathway and not other external factors. Interestingly, although mice did show increased rotational behaviour after amphetamine and not saline injections, this increase was not significant and therefore we were confident that the rotations that were observed in lesioned mice was a reflection of dopamine loss. Similar increased rotational activity has been previously reported (Jerussi & Glick., 1974), however, in this particular case, non-lesioned rats showed significantly higher rotations following amphetamine stimulation compared to
saline injection, suggesting that rats had an innate imbalance of the nigrostriatal dopamine system (Jerussi & Glick, 1974).

The increase in rotational activity observed in the non-lesioned 129 OLA mice could also reflect an inherent asymmetry of the nigrostriatal pathway. Alternatively, the increased rotations could be simply a result of amphetamine-induced enhanced locomotor activity. Regardless of the cause of these rotations, to be confident that rotations seen in lesioned animals are reflecting dopamine loss, either pre-lesion rotations as well as post-lesion rotations should be carried out, with the difference in rotation used as the final rotation score. Alternatively, one could lesion against spontaneous bias (i.e., if non-lesioned mice rotated to the right, then lesioning the left nigrostriatal pathway would result in rotations towards the left following amphetamine challenge. Any rotations to the left would then provide a more reliable index of the loss of dopamine).

6.4.3 MFB vs. Striatal lesions

The two different lesion models used in this study produced either near total (MFB) or partial (striatal) unilateral lesions of the nigrostriatal dopamine pathway. As a result there was a marked difference in rotations between the two groups, with MFB lesion animals responding to both amphetamine and apomorphine stimulation, while striatal lesion mice responded significantly more to amphetamine than apomorphine. When the two lesion groups were directly compared, the degree of amphetamine-induced rotations was generally higher for the partial lesion group, and significantly higher at 10mg/kg. In contrast, apomorphine-induced rotations were more pronounced in the MFB group compared to the striatal lesion group with doses of 2.5mg/kg and 5mg/kg producing significantly higher rotations.

Since at least 50% dopamine loss is necessary for amphetamine-induced rotations to be observed (Hefti et al., 1980a; 1980b; Hudson et al., 1993), it is unsurprising that both lesion groups showed rotations following amphetamine stimulation. It is somewhat surprising however, that amphetamine was more effective at producing rotations in the striatal lesion group when compared to the MFB lesion group. MFB lesions produce extensive and maximal dopamine depletions, and therefore it would seem logical that these lesions would result animals displaying better rotations than animals receiving striatal lesions. The reason why this does not occur is explained by
the severity of the MFB lesion. In addition to depleting the nigrostriatal dopamine pathway, MFB lesions also deplete the dopaminergic neurones of the ventral tegmental area (VTA), which project to the nucleus accumbens. Although the role of the nucleus accumbens is not involved in the direction of rotation, it is involved in locomotor activity (Kelly et al., 1983) and therefore influences the speed of rotations. This was clearly demonstrated by Brundin and colleagues who showed significant reduction in amphetamine-induced locomotor activity and rotational behaviour following bilateral 6-OHDA lesions of the nucleus accumbens. In addition, dopamine grafts into the lesioned accumbens significantly increased both of these motor behaviours (Brundin et al., 1987).

The decrease in locomotor activity following the lesioning of the VTA by MFB lesions may therefore explain why amphetamine causes greater rotational bias in striatal lesion animals when compared to MFB lesion animals.

The difference in apomorphine-induced rotational behaviour between the two lesion groups is explained by whether or not up-regulation and hypersensitivity of postsynaptic dopaminergic receptors occurs (Hefti et al., 1980a; 1980b; Hudson et al., 1993).

Since MFB 6-OHDA lesions produce near complete unilateral destruction of the nigrostriatal pathway, striatal post-synaptic dopamine receptors are up-regulated and become highly sensitive as a compensatory mechanism for dopamine loss. Since this global receptor hypersensitivity only occurs when more than 90% of the nigrostriatal system is destroyed (Hefti et al., 1980a; 1980b), global hypersensitivity will not occur in the lesioned striatum of the partial lesion model. Instead, only localised areas of post-synaptic receptor hypersensitivity will occur, with the majority of compensation being provided by increased dopamine synthesis from the remaining neurones (Hefti et al., 1980a). Because of this global hypersensitivity in the MFB lesion group, not only are low doses of apomorphine sufficient to induce rotations, but also, this lesion model is more responsive to apomorphine than the striatal lesion model.

While this would explain why the MFB model produced better rotations than the striatal model following apomorphine stimulation, hypersensitivity of receptors probably did not occur to a great extent, since low doses of apomorphine (0.01-0.05mg/kg) did not result in any rotational behaviour. In addition, most of the MFB lesions resulted in less than 90% dopamine cell loss. Therefore, the rotations observed
in MFB lesion mice were probably a combined result of increased numbers of post-synaptic dopamine receptor and increased receptor sensitivity. Similar to hypersensitivity, receptor up-regulation would be global in the striatum of the MFB model, but localised in the striatum of the partial lesion model. This combination of global receptor hypersensitivity and up-regulation would explain why significantly higher apomorphine-induced rotations were observed in the MFB lesion group compared to the striatal lesion group at relatively high drug doses (2.5 & 5mg/kg).

In addition, post-synaptic receptor up-regulation probably also occurred in the nucleus accumbens of MFB lesion mice, as previously shown following dopamine depletion of the nucleus accumbens (Brundin et al., 1987). Therefore the rotations observed in the MFB lesion group following apomorphine challenge was probably a collective result of increased excitation of the lesioned striatum and increased rotational drive initiated by the activation of the nucleus accumbens.

The combination of striatal rotational bias and the locomotor activity or ‘drive’ initiated by the accumbens probably explains why a shift in the dose-response curve for striatal lesions is observed. For example, at 2.5 & 5mg/kg amphetamine, MFB lesioned animals rotate less than striatal lesioned animals because although MFB lesions have larger rotational bias, they have less drive than striatal lesioned animals because of the lesioned accumbens. At 10mg/kg amphetamine, increased stimulation of all dopaminergic areas result in stereotypy in MFB lesioned animals and low levels of rotation. This does not occur in striatal lesioned animals because at this dose, although other dopaminergic regions are stimulated, there is maximal stimulation of the accumbens on both sides of the brain. This results in excessive drive which overshadows the other responses resulting in larger rotational responses than those observed at 5mg/kg, and a shift in the dose-response curve. However at 15mg/kg, although accumbens stimulation is still maximal, stimulation of regions outside the accumbens and lesioned dorsal striatum is excessive, and therefore stereotypy is induced and rotations decrease.

With apomorphine-induced rotations, at low doses (0.05mg/kg), no rotational behaviour was observed because hypersensitivity and up-regulation of post-synaptic dopamine receptors was insufficient. At 2.5 & 5mg/kg apomorphine however, because of the larger rotational bias and the larger drive (because of supersensitivity and up-regulation of dopamine receptors in the lesioned accumbens), rotations are
higher in the MFB lesion model than the striatal lesion model. At 10mg/kg apomorphine, although drive is high in the MFB lesion group, rotations are lower compared to 5mg/kg because there is lower rotational bias due to high-dose apomorphine stimulation of intact striata. However, in the striatal lesion model, although rotational bias will be smaller because of stimulation of the intact striata, apomorphine rotations are higher than those observed at 5mg/kg because the drive is increased due to high-dose apomorphine stimulation of the nucleus accumbens. This therefore causes a shift in the striatal lesion dose-response curve for apomorphine. At 15mg/kg apomorphine, striatal lesioned animals show decreased rotations because of increased stimulation of the intact striata which reduces rotational bias.

An alternative explanation for the reduced rotational behaviour at 10mg/kg apomorphine for MFB lesioned mice could be because of stereotypy. However, this probably did not occur since if stereotypical behaviour is induced at this dose, then one would expect stereotypy to be induced in the striatal model also, and this clearly does not occur.

6.4.4 Dopamine loss vs. Rotations

Unilateral 6-OHDA MFB lesions in mice have recently been used to produce a model of PD which shows good rotational asymmetry in response to amphetamine and apomorphine challenge (Iancu et al., 2005). However, while this model shows good rotational behaviour, less than 50% of the total number of animals that were lesioned in the study by Iancu et al., (2005) were selected to participate in behavioural tests. The reason why only a small proportion of animals were used was because the MFB lesion was variable, which meant that some animals had good lesions, whereas others did not. Consequently, only the animals with good lesions were chosen.

Histological examination of the MFB lesioned animals in our study also shows that MFB lesions are variable. In contrast, the striatal lesions show more reliable and consistent dopamine loss. This difference in lesion reliability is reflected in rotational behaviour, where striatal lesion animals rotate better (following amphetamine challenge) than MFB lesion animals. Like the study by Iancu, we could also have selected a proportion of MFB lesion mice that showed good rotational behaviour, and only used these animals in the rotation tests. However, while this would have certainly increased the average rotational bias of this lesion group, it would not have
reflected the significant inconsistencies of dopamine loss produced by the MFB lesion. The level of inconsistency of the MFB lesions is extremely relevant when attempting to reproduce this model since it would mean that more animals than required would need to be lesioned, so that sufficient numbers of animals would display good lesions. The level of inconsistency of the MFB lesions is therefore unacceptable, and whenever possible striatal lesions should be used to produce a model of PD in mice.

Since only 7 mice received striatal lesions compared to 24 mice which received MFB lesions, it can be argued that striatal lesions do not show much lesion variability because of the few animals lesioned. Although this is a valid argument, the reason why MFB lesions are so variable is because the MFB is relatively small and is therefore difficult to target. The striatum on the other hand, is easier to target and therefore it is unlikely that such variability would result from striatal lesions.

In summary, the 129 OLA mouse strain exhibits rotational bias following unilateral 6-OHDA lesioning of either the medial forebrain bundle (MFB) or the striatum. Lesions of the MFB model produced rotations that are responsive to both amphetamine and apomorphine with peak rotations occurring at a drug dose of 5mg/kg for both drugs. The striatal lesion model however, is more responsive to amphetamine than apomorphine-induced rotations with peak rotations being induced at a dose of 10mg/kg for both drugs.

There was little correlation between dopamine loss and rotations following amphetamine and apomorphine stimulation in MFB lesioned animals. Striatal lesioned animals were more reliable since more consistent lesions, more consistent rotation, and stronger correlations were observed especially with amphetamine rotations. Striatal lesions also showed less variability in rotations particularly following amphetamine stimulation confirming the reliability of the striatal lesion model.

The data reported here suggest that the optimal model for Parkinson's disease in the 129 OLA mouse strain is produced following striatal lesions, which can be measured most effectively using drug-induced rotation with an optimal dose of 10mg/kg amphetamine. Although the striatal 6-OHDA lesion model is not novel, the model
established here is fundamentally different and better than previous mouse models since the conditions which produce optimal rotational behaviour have been defined.
Chapter Seven

Experiment Five - Functional recovery of rotational asymmetry in unilateral 6-OHDA-lesioned mice

Summary
This chapter examines whether the recently developed 6-OHDA-lesion mouse model of Parkinson’s disease (see chapter six), is reliable in assessing the functional capacity of transplanted dopamine neurones.

Mice with partial, unilateral lesions of the nigrostriatal pathway, which showed good rotational behaviour following 10mg/kg amphetamine challenge, received intrastriatal grafts of E13 mouse mesencephalic dopamine neurones. Rotational behaviour of these mice was then assessed at 2, 4 and 6 weeks post-transplantation. Results showed no substantial difference in rotation at 2 weeks post-transplantation, however a significant reduction in ipsilateral rotational bias was observed at 4 & 6 weeks post-transplantation when compared to pre-graft scores. Histological examination of the dopamine grafts showed that transplanted cells formed large grafts which contained in excess of 1200 dopamine neurones. The absence of any attenuation of rotational asymmetry in non-transplanted lesioned mice over the 6 week testing interval confirmed that the behavioural recovery seen in grafted group was attributable to striatal reinnervation by grafted dopamine neurones and not by spontaneous recovery of the lesioned striatum.

These results show that stable, unilateral 6-OHDA lesions can be well established in mice, and that this model is suitable for assessing the functional ability of mouse-derived ES and other mouse-derived cell lines without the need for immunosuppression.
7.1 Introduction

Intrastriatal transplantation of embryonic dopamine neurones into the dopamine-depleted striatum has provided strong evidence that neurotransplantation strategies are effective at reinnervating the lesioned striatum. This evidence has not only come from animal models where improvements in lesion-induced behavioural deficits such as rotational asymmetry have been observed (Perlow et al., 1979; Björklund & Stenevi, 1979; Dunnett et al., 1983), but also from clinical trials where improvements in motor control have been demonstrated following transplantation of human foetal mesencephalic tissue in Parkinson’s disease (PD) patients (Wenning et al., 1997; Lindvall et al., 1990; 1992; Kordower et al., 1995; Kopyov et al., 1997).

However, while cell replacement therapies are clearly effective, the clinical application of this procedure is limited by the nature of the tissue used. Alternative tissue sources that can form dopamine neurones and are capable of integrating with the brain are therefore required if neurotransplantation strategies are going to succeed in providing treatments for PD. To date, most success has come from using embryonic stem cells (ES cells). These cells have not only shown the generation of dopamine neurones in vitro (Lee et al., 2000; Perrier et al., 2004), but they have also shown to alleviate rotational bias when transplanted into rat models of PD (Kim et al., 2002; Björklund et al., 2002). Assessing the potential of human or mouse-derived ES cells to develop into functional dopamine neurones via transplantation into rats models of PD, is however, far from ideal since rats receiving the xenografts require immunosuppression to avoid graft rejection. This immunosuppression is normally achieved by the administration of the drug Cyclosporin A (CsA), which suppresses the immune response by inhibiting T-cell activation (Kadereit et al., 2001). While CsA promotes xenograft survival (Brundin et al., 1985), its use is problematic because not only is prevention from cell rejection not absolute (Pedersen et al., 1997), but also, CsA treatment is associated with toxic side effects such as hepatotoxicity (Deters et al., 1997). Rats treated with CsA also have increased health risks, presumably because of the suppression of the immune system, and show a decline in their general health with notable weight loss and reduced life span. These effects of this immunosuppressant can be fatal as illustrated in many studies, for example, where a number of CsA-treated rats receiving grafts of human-derived neural
progenitor cells have died due to side effects of CsA treatment (Svendsen et al., 1997a).

Interestingly, continued injections of CsA into unilateral 6-OHDA-lesioned rodents have also been shown to elevate striatal dopamine levels, perhaps by having neuroprotective effects or by promoting regeneration of nigral-striatal dopamine neurones (Matsuura et al., 1996; 1997). This property of CsA is extremely relevant when assessing the functional capacity of cells to restore behavioural deficits, since any recovery of grafted cells will be a result of the combination of the cells’ potential to generate functional dopamine neurones and the effect of CsA-induced increase in striatal dopamine. These problems associated with the use of CsA means that if possible, treatment with CsA should be avoided, which could readily be achieved if the species of the donor and recipient are the same. The most obvious choice would be to transplant rat-derived ES cells into the well established rat model of PD. However, the generation of ES cells from rats is itself proving problematic. The other alternative for exploring the differentiation potential of embryonic stem cells, without immunosuppression, is to graft mouse embryonic stem cells into the mouse brain. Measuring rotational bias in mice is however difficult because in spite of a couple of brief reports (Brundin et al., 1986; Iancu et al., 2005) the 6-OHDA lesion model has not been fully characterised in mice.

The recently developed 6-OHDA-lesion mouse model of PD shows appropriate rotational bias following amphetamine stimulation (see chapter six). As a first step to validating the reliability of this model to assess the functional capacity of mouse-derived ES and other cell lines (such as the Nurrl-overexpressing neural stem cells), we have assessed behavioural recovery following intrastratal transplantation of primary VM neurones. Grafts of E13 mouse VM tissue were transplanted into the lesioned striatum of mice and amphetamine-induced rotational behaviour was assessed at 2, 4 and 6 weeks post-transplantation. A significant reduction in rotations was observed at 4 & 6 weeks post-transplantation indicating reinnervation of the striatum.

This chapter demonstrates that our mouse model of PD is a reliable animal model, which shows appropriate behavioural recovery following transplantation of functional dopamine neurones. This model provides a tool to efficiently assess the potential of mouse-derived ES cells and other mouse-derived cell lines to provide functional recovery without the need for immunosuppression.
Chapter Seven

7.2 Experimental Procedure

Adult female 129OLA mice (~25g) received unilateral 6-OHDA lesions of the right midstriatum (see chapter two, section 2.7.1). After full recovery from surgery, mice were habituated to experimental conditions for 10 minutes before 10mg/kg d-amphetamine-induced rotational testing commenced. Rotations lasted 90 minutes and were assessed in a closed room to avoid any environmental disturbance (see chapter two, section 2.8.2.1). Only mice showing good rotational behaviour (at least 6 net ipsilateral turns per minute) were selected for this experiment. Lesioned mice were split into 2 equal groups (2 x n=8), with one group receiving transplants of E13 mouse VM cells into the right striatum (see chapter two, section 2.7.2), and the other group remaining as the non-transplanted lesion group. As a control, non-lesioned mice were also tested for rotation bias (n=10). The grafted group, non-transplanted lesion group and non-lesion groups underwent amphetamine-induced rotation at 2, 4 & 6 weeks post-transplantation. After behavioural testing, animals were sacrificed, brains were isolated and the striatal dopaminergic grafts were analysed (see chapter two, section 2.9.4).

Statistical tests of analysis of variance (ANOVA) were analysed using the statistical package Genstat v7.2, with further analysis of the results being achieved by using the Neuman Keuls t-test.
7.3 Results

7.3.1 Amphetamine-induced rotations

The effect of E13 mouse VM grafts on unilateral 6-OHDA lesion-induced rotational asymmetry can be seen in figure 7.1, along with the rotational behaviour of lesioned and non-lesioned mice over a 6-week period. Rotational behaviour was only assessed for 6 weeks since this time period was sufficient for grafts to reverse 6-OHDA lesion-induced rotational deficits.

Mice receiving grafts of VM tissue (n=8), showed a significant decline in rotational bias at 4 (NK: t(3,3)=13.125, p<0.01) and 6 weeks (NK: t(4,3)=13.546, p<0.01) post-transplantation when compared to pre-graft scores (Fig. 7.1). No difference was observed however between pre-graft rotations and rotations 2 weeks post-transplantation (NK: t(2,3)=0.937, n.s).

Lesion only mice (n=8) showed good, stable rotational behaviour with no difference in rotational scores throughout 6 weeks of testing. When compared to rotations in mice receiving grafts, a significant difference was observed at 2 (NK: t(2,2)=7.707, p<0.05), 4 (NK: t(2,2)=17.099, p<0.01) and 6 weeks (NK: t(2,2)=17.892, p<0.01) post-transplantation (Fig. 7.1).

As expected, non-lesioned mice (n=10) showed no significant rotational behaviour (Fig. 7.1). Statistical analysis of the data confirms a significant difference in rotational behaviour with groups (F(2,103)=83.92, p<0.001).

TH immunohistochemistry of all three groups is shown in Fig. 7.2, with grafts of dopamine neurones clearly visible (Fig. 7.2D) Using the Abercrombie correction formula, grafts were calculated to contain in excess of 1200 dopamine neurones.
Figure 7.1 Amphetamine-induced rotational behaviour. A significant difference in rotational bias with groups is observed (p<0.001), with VM grafted groups showing a reduction in rotation at 4 and 6 weeks post-transplantation when compared to pre-graft rotation scores. Lesion only mice show stable rotations throughout the 6-week testing period and non-lesioned rats show no significant rotational bias. **a p<0.01 vs. pre-graft scores, **b p<0.01 vs. lesion only group, *b p<0.05 vs. lesion only group. Data expressed as mean ± S.E.M.
Figure 7.2 TH immunohistochemical staining in brain sections moving rostral-caudal from left to right through the levels of the striatum and the substantia nigra. Lesion groups show partial depletion of TH staining on right hand side, in both the striatum and the substantia nigra (A). Control groups show equal staining for TH in the striatum and substantia nigra on both sides of the brain (B). VM Graft can be clearly seen in the striatum (C). Scale bar represents 2 mm. Higher magnification of graft (in C) shows the presence of TH neurones (D).
7.4 Discussion

Rotational asymmetry caused by unilateral depletion of striatal dopamine, and its subsequent behavioural recovery following intrastrital transplantation of dopamine neurones is a model that has been well characterised in rats, but not so well established in mice. With the successful in vitro generation of mouse-derived dopamine neurones (Lee et al., 2000; Kim et al., 2003), we believe the requirement for a reliable mouse model is essential so that the functional potential of these cells can be efficiently assessed without the added complications of immunosuppression.

The need for a mouse model of Parkinson’s disease has also recently been addressed elsewhere and unilateral 6-OHDA lesioned mice showing appropriate behaviour have been established (Iancu et al., 2005). However, while this study shows that drug-induced rotations, cylinder and rotarod tests are the most sensitive tests for reflecting dopamine loss, functional recovery of these tests following transplantation of dopamine neurones was not assessed. This chapter examined whether our recently developed 6-OHDA-lesion mouse model of Parkinson’s disease, which shows good rotational bias as a result of significant dopamine loss, can show behavioural recovery following intrastrital transplantation of mouse dopamine neurones.

Prior to transplantation, unilateral 6-OHDA lesioned mice showed good rotational behaviour following 10mg/kg amphetamine stimulation with an average pre-transplantation score of 12-13 ipsilateral turns per minute. Little change in rotational bias was observed at 2 weeks following grafting, however a significant reduction in rotations was observed at 4 & 6 weeks post-transplantation, indicating reinnervation of the lesioned striatum by grafted dopamine neurones (Björklund et al., 1980; Dunnett., 1992). Although overcompensation of motor response was not observed when looking at the average rotation scores of the whole group, two animals did show complete reversal of rotational bias and contralateral rotations. Why this was not demonstrated for all animals is unclear, but perhaps dopamine grafts required longer than 6 weeks to mature.

Analysis of striatal transplants revealed large surviving grafts containing over 1200 dopamine neurones. Since 500,000 cells were transplanted and approximately 10% of cells stained immunopositive for TH in vitro (data not shown), the survival rate of grafted dopamine neurones is equivalent to approximately 2-3%, which is consistent
with the typically low survival rates of grafted rat dopamine neurones previously reported (Brundin et al., 2000).

The rotational behaviour of non-transplanted mice was also measured and showed consistently high ipsilateral turns throughout the 6-week testing period. This confirmed that the attenuation of rotational asymmetry was solely because of dopamine graft-induced reinnervation of the dopamine-depleted striatum and not because of spontaneous recovery of the striatum, which has been previously reported in mice, albeit following MPTP-induced lesions (Iancu et al., 2005 and references therein). The absence of any spontaneous recovery also demonstrates that 6-OHDA lesions of the striatum show long-term stability, which is an essential requirement for a reliable animal model of PD. As expected, non-lesioned mice showed no substantial rotational bias throughout the 6-week testing period, although a gradual increase in rotations of non-lesioned animals was observed at 4 & 6 weeks post-transplantation. This increase may reflect a conditioned response to amphetamine administration resulting in hyperactivity of mice, however, this increase in rotational behaviour was not significantly different from pre-graft levels and therefore is not of great concern.

In summary, we provide evidence that functional recovery of lesion-induced behavioural deficits can be achieved in unilateral 6-OHDA-lesioned mice following intrastriatal transplantation of mouse dopamine neurones. This model is therefore suitable for assessing the functional potential of mouse-derived cell lines without the need for immunosuppression. This mouse model was initially established to assess the potential of mouse-derived ES cells to generate functional dopamine neurones in vivo. These cells are currently being generated by Professor Austin Smith’s laboratory at Edinburgh University, but due to technical and practical difficulties in cell production, this study has been delayed. However, when these cells become available, we have a reliable, efficient model in place which is ready to assess the potential of these cells.
Chapter Eight

General Discussion

Neurotransplantation is an effective strategy for treating neurodegenerative disorders such as Parkinson's disease, however, its clinical application is limited by the absence of a suitable donor source of neurones. The ability of stem cells to generate neurones may provide a solution to this problem, and has consequently been the focus of this research. This thesis attempted to assess the potential of embryonic, foetal and adult neural stem cells to provide a source of neurones suitable for transplantation for Parkinson's disease. Unfortunately, embryonic stem cells could not be obtained, and therefore the results described here only reflect the neurogenic capacity of foetal and adult neural progenitor cells.

This chapter outlines the important parameters which affect neuronal differentiation of progenitor cells, discusses the importance of increasing dopamine survival following transplantation, and reviews the current status of embryonic, foetal and adult stem cell research in terms of providing a treatment for Parkinson's disease. In addition, the case for a 6-OHDA-lesion mouse model of Parkinson's disease is evaluated, and finally, based on the results from this thesis, the chapter ends with a discussion concerning the directions that future research studies must concentrate on if foetal and adult progenitor cells are going to be considered for clinical transplantation programmes.

8.1 Factors affecting neuronal differentiation of neural stem cells

8.1.1 Donor age

Over the past decade, an enormous amount of research has focused on generating sufficient numbers of neurones of appropriate phenotypes from neural stem cells. While this research has clearly accelerated our knowledge on the fundamental aspects of neural stem cell behaviour, the field of stem cell research remains inconsistent, with huge variability in the protocols used between different laboratories to dissect, prepare, culture and/or transplant stem/progenitor cells (Gage., 2000). Such variability
means that comparing studies from different laboratories is difficult, and those comparisons that are made are often based on the assumption that neural stem/progenitor cells behave identically. Since one common variable is the donor age from which neural stem/progenitor cells are isolated, the \textit{in vitro} neurogenic potentials of cells isolated from different donor ages were examined in order to determine whether this factor had any effect on progenitor cell behaviour. The results from this study were conclusive and showed that neural progenitor cells from different donor ages had distinct neurogenic potentials, a finding which is of particular importance in terms of research studies since it means that direct comparisons between different studies cannot be made unless donor age are the same. In addition, some of the other variables such as the type of enzyme used in cell dissociation and the growth media used in progenitor cell proliferation may also have pronounced effects on progenitor cell behaviour, particularly since these factors have been previously shown to affect the properties of other cells (Barker \textit{et al}., 1995; Brundin \textit{et al}., 2000). The experimental variations used to investigate stem cells are therefore causing unnecessary complications, and for this reason, research would perhaps benefit from standardising all experimental conditions so that comparisons between studies can be easily achieved.

The effect of donor age is, of course, also relevant in terms of the clinical application of these cells in the treatment of neurodegenerative disorders. In this case, selecting the age of donor tissue which gives optimal differentiation of specific neuronal phenotypes, is imperative for treating diseases such as Parkinson’s and Huntington’s disease. Interestingly, the optimal age for the generation of dopamine neurones from rat VM tissue has been reported to correspond to approximately embryonic day 13-15 (Barker \textit{et al}., 1995; Brundin \textit{et al}., 2000), resulting in the majority of transplantation studies using E14 VM tissue. However, results from a study presented in this thesis are in contrast to these findings and suggest that optimal numbers of dopamine neurones are generated when using E12 VM tissue.

\textit{8.1.2 Cell proliferation}

One of the properties of neural stem cells is their potential to undergo long-term proliferation whilst retaining the ability to generate neurones. In theory, this property is very appealing since it would provide a continuous supply of neurones, which could potentially be used for transplantation. However, based on the results in this thesis,
and from data published elsewhere (Smith et al., 2003), it is clear that even short periods of in vitro proliferation result in rat progenitor cells losing their ability to differentiate into neurones. Since there is little data comparing the effects of prolonged proliferation on the differentiation of progenitor cells, and evidence of species-specific properties exist (Svendsen et al., 1997), it is currently unclear whether this finding is a general characteristic of progenitor cells, or a species-specific property of rat progenitor cells. The generation of neurones from long-term expanded human progenitor cells (Carpenter et al., 1999) suggests that the loss of the neurogenic potential of rat progenitor cells is a species-specific characteristic, or alternatively, a result of the culture conditions being inadequate to support neuronal differentiation following cell expansion. However, on closer inspection, the data by Carpenter et al, also shows a reduction in the neurogenic potential of human progenitor cells after proliferation, albeit following much longer periods in culture, thus indicating that the loss of neurogenic potential could be a general property of progenitor cells. The capacity of expanded progenitor cells to generate neurones therefore merits further investigation since this property is crucial to the success of these cells in providing an alternative to the use of foetal tissue in neurotransplantation.

8.2 Increasing the survival of transplanted dopamine neurones is imperative for the success of cell replacement strategies for Parkinson’s disease

One of the problems associated with cell replacement strategies for Parkinson’s disease is the inadequate survival of dopamine neurones following transplantation. In experimental models and clinical trials, only 5-10% of transplanted embryonic dopamine neurones have been reported to survive (Dunnett., 1992; Björklund., 1992). Similarly, the survival rate of functional dopamine neurones derived from expanded neural progenitor cells have also been shown to be typically low (Brundin & Björklund., 1998). This insufficient survival is partly the reason why dopamine grafts are unable to completely reverse all behavioural deficits, and is consequently a major limiting factor for the use of these cells in clinical trials of Parkinson’s disease. In an attempt to increase dopamine cell survival, embryonic dopamine neurones were transplanted into unilateral 6-OHDA lesioned rats in a solution containing the antioxidant ascorbic acid. Although no difference in the recovery of rotational behaviour was observed in these animals when compared to animals receiving
standard dopamine grafts, analysis of the transplants 6 weeks post-transplantation revealed that neurones treated with ascorbic acid formed grafts containing significantly more dopamine neurones compared to grafts of standard dopamine cells. While the increased presence of dopamine neurones is likely to reflect enhanced dopaminergic survival, it is also possible that ascorbic acid increased dopaminergic expression via other mechanisms i.e. by increasing dopaminergic differentiation. Although the precise mechanisms involved in this increase of dopamine neurones remain elusive, it is clear that reducing dopamine death following transplantation, perhaps by the administration ascorbic acid, alternative antioxidants or even growth factors (Zawada et al., 1998), is an absolute prerequisite if dopamine replacement therapies are going to provide an efficient treatment for Parkinson’s disease. Only when this is achieved will the use of neural progenitor cell-derived dopamine neurones be of any value in clinical trials.

8.3 Embryonic, foetal and adult stem cells – which cells are best?

The capacity of embryonic, foetal and adult neural stem cells to generate neurones has been discussed extensively throughout this thesis. It is clear that while the use of adult neural stem cells might be the preferred cell type, embryonic stem cells have a far greater potential for generating functional dopamine neurones, and are therefore currently the strongest candidate for providing a source of neurones in cell replacement therapies for Parkinson’s disease. The use of adult neural stem cells is particularly favourable because their use is not associated with the ethical concerns that surround the use of embryonic and foetal tissue. It is also possible that these cells can be taken for autologous transplantation, a procedure that will avoid the complications associated with immunosuppression and tissue rejection. In contrast, embryonic stem cells are probably the least favoured cell type because not only is the use of these cells seen by some as ethically unacceptable, but also, their use is associated with serious safety concerns since they have the potential to generate tumours following transplantation. Unfortunately however, the generation of functional dopamine neurones from adult neural progenitor cells has not been reported and therefore the use of these cells is currently unfeasible in transplantation strategies. Foetal neural progenitor cells have fared little better with only a few studies reporting the generation of functional dopamine neurones. The most encouraging results of functional dopamine generation have come from
embryonic stem cells, however considering their ethical and safety concerns can these cells be truly successful in transplantation strategies for Parkinson's disease? The safety issues associated with the use of embryonic stem cells will be removed if the potentially dangerous tumour-forming undifferentiated cells can be efficiently eliminated. This is an area that has been the focus of much research and methods have been developed which can efficiently select and destroy such cells (Schuldiner et al., 2003; Fareed & Moolten., 2002). However, while the safety element surrounding the use of embryonic stem cells can be solved, the ethical debate that is related to their use will always be present and is an issue for social consensus rather than scientific determination. Embryonic stem cell research is currently in a position similar to what was seen with the emergence of in vitro fertilisation (IVF) techniques in the late 70's. IVF treatment was seen as unethical since the procedure generates more embryos than are required, and therefore results in some embryos being destroyed. However, since this procedure enables children to be born to infertile couples, it is a technique that is now widely accepted. Similarly, destroying embryos left over from IVF procedures to provide a treatment for Parkinson's disease will hopefully become ethically acceptable.

8.4 Do we need a 6-OHDA-lesion mouse model of Parkinson's disease?

The unilateral 6-OHDA-lesion model of Parkinson's disease is an efficient model for assessing the functional potential of dopamine neurones. However, while this model has been extensively used in the rat, it is surprising that this model has not been more extensively used in mice, especially when considering the advances made in mouse embryonic stem cell research and developments of genetic mouse experimental models. One possible reason for the absence of this model in mice might simply be because the rat model is more than sufficient to assess the functionality of cells, and therefore the requirement for a mouse model is not necessary. In addition, lesioning of the dopaminergic nigrostriatal pathway in mice is more difficult when compared to rats, since the medial forebrain bundle (MFB) is relatively small in size. Therefore accurately targeting and destroying this structure is difficult and consequently results in mice often showing variable levels of dopamine cell loss. Considering the success of the rat model and the unreliable lesions in mice, would a 6-OHDA-lesion mouse model of Parkinson's disease be of any benefit in stem cell research?
In this thesis, a novel 6-OHDA-lesion mouse model based on targeting lesions in the area of the striatal terminals rather than in the MFB has been established, which accurately reflect dopamine loss and show appropriate behaviour following reinnervation of the denervated striatum. We believe that the requirement for this model is highly warranted so that the potential of mouse-derived dopamine neurones can be efficiently assessed without the complications of immunosuppression that are currently associated with the use of the rat model. This mouse model of PD will undoubtedly be a valuable tool in measuring the functional capacity of mouse-derived cell lines.

8.5 Conclusions and further considerations

The results presented in this thesis have addressed some of the important parameters which affect the differentiation potential of neural progenitor cells, and while it is apparent that both foetal and adult progenitor cells have the capacity to generate neurones, it is clear that future studies must focus on a number of issues if these cells are going to provide a source of dopamine neurones for cell replacement therapies in Parkinson's disease. These issues mainly centre around increasing both dopamine differentiation and dopamine cell survival following transplantation, two factors which are of equal importance and will ultimately dictate the successful clinical use of any potential cell replacement-based therapy.

With foetal neural progenitor cells increasing dopamine differentiation is imperative, since even following relative short periods of expansion these cells fail to generate dopamine neurones. Although progress in this area is currently being made with a number of studies reporting the generation of dopamine neurones following cell expansion, these studies are limited and cell expansion has not been extensive. The focus of research must therefore be to increase dopamine differentiation following long-term progenitor cell proliferation. Of course, dopamine differentiation will not be beneficial if the survival of dopamine cells following transplantation is poor, and for this reason dopamine cell survival must also be enhanced. Although ascorbic acid treatment was successful in promoting dopamine graft survival in a study reported in this thesis, perhaps ascorbic acid in combination with other antioxidants (Love et al., 2002), growth factors (Zawada et al., 1998) or even different cell preparation techniques (Barker et al., 1995) could reduce the death of dopamine cells even further.
following transplantation. If these two goals are met, foetal neural progenitor cells will be an excellent source of neurones for transplantation, since fewer cells need to be transplanted and larger surviving grafts would be observed, which would perhaps result in improved recovery of function.

With adult neural progenitor cells, it is evident from the results in this thesis and from the literature that neuronal differentiation is low and dopamine differentiation is non-existent. The main priority of research is therefore to increase neuronal yield of these cells and determine whether or not adult progenitors can generate dopamine neurones. Achieving these goals will be a major step forward in adult neural progenitor cell research, and only when this is done can the use of these cells be considered in clinical transplantation programmes for Parkinson's disease.

The results discussed in this thesis have highlighted a number of important parameters which affect the differentiation potential of neural stem cells, and will accelerate the progress of further studies researching stem cell behaviour. From the current research it is clear that the factors which govern the behaviour of these cells are still not fully understood, and therefore a wealth of research has yet to be done before the clinical application of neural progenitor cells can be considered.
Appendix 1: Recipes for solutions and culture media

**General Buffers**

*Phosphate-buffered saline (PBS)*

Basic 0.1M solution used as an isotonic buffer in immunocytochemistry. One litre PBS is composed of: 9g sodium chloride, 11.46g disodium hydrogen orthophosphate and 7.96g sodium dihydrogen orthophosphate. Make up to one litre with distilled water. Adjusted to pH 7.4 with hydrochloric acid.

*TRIS-buffered saline (TBS)*

Used as a basic histological buffer. To one litre of distilled water is added: 12g Trizma base and 9g sodium chloride. Adjusted to pH 7.4 with concentrated hydrochloric acid.

*Triton X TBS (TXTBS)*

To 250ml TBS add 500μl Triton X-100. Adjusted to pH 7.4 with concentrated hydrochloric acid.

*TRIS non-saline solution (TNS)*

To one litre of distilled water, 6g Trizma base is added. Adjusted to pH 7.4 with concentrated hydrochloric acid.

*TRIS-buffered saline + sodium azide (TBZ)*

This solution prevents microbial growth and allows long-term storage of histological specimens. To 500ml of TBS add 2.5ml of 20% sodium azide.

**Solutions used in perfusion and fixative protocols**

*Pre-wash*

For one litre of pre-wash add: 18g disodium hydrogen phosphate (dehydrate), 9g sodium chloride and make up to one litre with distilled water. Adjust to pH 7.4 with orthophosphoric acid.
4% Paraformaldehyde (PFA)
The basic fixative for tran-cardial perfusion of animals. For five litres add: 90g disodium hydrogen phosphate, 45g sodium chloride, and one litre of 20% PFA (1kg PFA + 5 litres distilled water). Make up to five litres with distilled water and adjust to pH 7.4 with orthophosphoric acid.

Culture media
The recipes for the growth and differentiation media used for embryonic and adult tissue can be found in chapter two, sections 2.4.2 and 2.5.2. All media is filter sterilised and stored at 4°C.

Dissection media
For 45ml dissection media add: 43ml DMEM/F12, 1ml 30% glucose (30g glucose in 100ml distilled water), 0.75ml 7.5% NaHCO3 (Gibco) and 0.25ml 1M HEPES (Gibco). Filter sterilise and store at 4°C.

0.05% DNase solution
To 5mg DNase (Sigma), add 10ml dissection medium. Filter sterilise and store at -20°C.

0.1% Trypsin solution
To 10mg trypsin (Worthington Biochemical Corporation), add 10ml DNase solution. Filter sterilise and store at -20°C.

Appendix 2: Suppliers

Sigma Poole, Dorset, UK
Gibco Paisley, Scotland, UK
R & D Systems Abingdon, Oxon, UK
Worthington Biochemical Corporation Freehold, New Jersey, U.S.A
DAKO Glostrup, Denmark


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