Factors influencing the survival, growth and development of central nervous system neurons in the fetal rat and human

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"If you want to build a ship, don't drum up people together to collect wood and don't assign them tasks and work, but rather teach them to long for the endless immensity of the sea."

– Antoine de Saint Exupery

Dedication

This thesis is dedicated to the memory of my mother, Stella Chalmers, who unforgettably touched the lives of everyone she met and whose all too short time on earth will be forever appreciated, remembered and cherished.

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Abstract

This thesis examines factors affecting the *in vivo* and *in vitro* survival, growth and development of CNS neurons from both rat and human. Target cells exert profound effects on projection neurons, as revealed by loss of tyrosine hydroxylase immunoreactive neurons in the ipsilateral substantia nigra after ibotenic acid lesioning of the striatum, the main target of these neurons. Grafting of fetal striatal primordia to the lesioned striatum resulted in a slight decrease in this effect, suggesting that striatal cells provide a trophic influence on the dopaminergic SN neurons. Retrograde bead labelling studies indicate that six weeks post-lesion, an increased number of bead-labelled cells do not express tyrosine hydroxylase immunoreactivity; such cells may be impaired but living dopaminergic striatal-projecting SN neurons.

The trophic support provided by the striatum may be in the form of protein trophic factors. Among the specific growth factors I examined *in vitro*, brain derived neurotrophic factor and basic fibroblast growth factor with heparin enhanced the number of surviving human fetal ventral mesencephalic dopaminergic neurons.

The dopamine precursor, L-dopa, has been suggested to have toxic effects on dopaminergic neurons *in vivo* and *in vitro*. In the experiments described here, however, L-dopa at drug dosages comparable to human therapy for Parkinson's disease had no toxic effect on either human or rat dopaminergic cells *in vitro*, or on fetal rat ventral mesencephalic grafts *in vivo*.

Progenitor cells from the fetal human forebrain can propagate in a growth-factor supplemented medium, and can subsequently be induced to express a variety of glial and neuronal phenotypes including dopamine. These cells exhibit a less than 48 hour cycle time as indicated by bromodeoxyuridine incorporation. These cells could be useful for pharmacological studies, examination of growth factors, transfection of desired genes or transplantation.

Abbreviations and Symbols

5-HT - serotonin

6-OHDA - 6-hydroxydopamine

AADC - aromatic acid decarboxylase

BDNF - brain-derived neurotrophic factor

bFGF - basic fibroblast growth factor

BrdU - bromodeoxyuridine

ChAT - choline acetyl-transferase

CMF-HBSS - calcium- and magnesium-free Hanks balanced salt solution

CNS - central nervous system

CPu - caudate putamen

DAB - diaminobenzidine

DIC - differential interference contrast

DIV - days in vitro

DMEM - Dulbecco's modified Eagle medium

DNase I - deoxyribonuclease I

EGF - epidermal growth factor

FBS - fetal bovine serum

FITC - fluorescein

GAD - glutamic acid decarboxylase

Gal-C - galactocerebroside

GFAP - glial fibrillary acidic protein

HBSS - Hanks balanced salt solution

IA - ibotenic acid

IGF-I - insulin-like growth factor I

IGF-II - insulin-like growth factor II

IgG - immunoglobulin

L-dopa - L-dihydroxyphenylalanine

MPTP - 1-phenyl-4-methyl-1,2,3,6-tetrahydropyridine

NGF - nerve growth factor

NGF-R - nerve growth factor receptor

PCM - progenitor cell medium

PCM-E - progenitor cell medium with epidermal growth factor

PCM-EI - progenitor cell medium with epidermal growth factor and insulin-like growth factor II

PD - Parkinson's disease

PFA - paraformaldehyde

SN - substantia nigra

SNc - substantia nigra pars compacta

SNr - substantia nigra pars reticulata

sub-P - substance P

TH - tyrosine hydroxylase

TT - tetanus toxin

VM - ventral mesencephalon

VTA - ventral tegmental area

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1.0 Factors That Affect Growth and Development of CNS Cells From Human and Rat

The majority of cells of the adult central nervous system (CNS) are highly differentiated post-mitotic cells. During development, they are directed by genetic and epigenetic influences and factors that modulate gene expression which leads to their final fully differentiated state. Developmental processes must be highly regulated in order to ensure appropriate and successful differentiation. Such processes include proliferation, migration from the site of cell division to the cells final destination, chemical and anatomical differentiation and synaptogenesis.

The fate of some invertebrate neurons is determined by the number and direction of cell divisions which sequester specific RNA and proteins to appropriate daughter cells. In the mammalian brain, however, environmental interactions in combination with the specific genetic program are required to establish the fate of the developing cell. Environmental factors, including growth factors, neurotransmitters, hormones and cell surface molecules are released or produced by target or supporting cells guiding the cell to its ultimate fate. These factors exert their influence by binding to cell surface receptors which transduce the message across the plasma membrane and ultimately influence gene expression. The developing cell is therefore particularly sensitive to its environment and these early interactions are crucial.

1.1 Influence of Growth Factors on Neural Development

Growth factors which have been shown to influence the growth, survival, differentiation, proliferation, synapse formation, migration, outgrowth and death of CNS cells include the neurotrophins, represented by NGF, BDNF, NT-3 and others; fibroblast growth factors (FGF), which include acidic FGF and basic FGF and others (Deloulme *et al.*, 1991; Sensenbrenner, 1993); epidermal growth factor (EGF) (Reynolds and Weiss, 1992); insulin-like growth factors (IGF-I and IGF-II) (Knusel *et al.*, 1990); platelet derived growth factor (Othberg *et al.*, 1995); glial cell-line derived neurotrophic factor (GDNF) (Lan *et al.*, 1993) and others. These growth factors exert their effects by the activation of transmembrane receptors which regulate the activity of cellular proteins or genetic material. I have chosen to examine the effects of a specific subset of growth factors including EGF, BDNF, bFGF, IGF-I and IGF-II, chosen on the basis of availability and previously published data (cited above) indicating specific effects on dopaminergic neurons and proliferating progenitor cells in defined species.

The heparin binding factors (Burgess and Maciag, 1989) exist in at least 9 different forms, including acidic FGF (aFGF or FGF1), basic FGF (bFGF or FGF2), *int-2* (FGF3), keratinocyte growth factor (FGF7) and androgen-inducible growth factor (FGF9) and exert their effects on a variety of cell types including some CNS neurons and glia (Dickson and Peters, 1987; Delli Bovi *et al.*, 1987; Zhan *et al.*, 1988; Marics *et al.*, 1989; Tanaka *et al.*, 1992; Miyamoto *et al.*, 1993). These proteins exhibit strong affinity for heparin and share a highly conserved core

sequence of amino acids. Lee *et al.* (1989) have described a high affinity receptor for bFGF and revealed it to be a typical tyrosine kinase transmembrane receptor. This discovery was soon followed by the identification of low affinity receptors, cell surface heparan sulfate proteoglycans (Kiefer *et al.*, 1990; Bernfield *et al.*, 1992) which were initially thought to be involved only in the sequestration of the growth factor but were later shown to be required for the appropriate delivery of the factor to its high affinity receptor (Ornitz *et al.*, 1992; Rapraeger *et al.*, 1991). The interactions of these receptors may serve to mediate the specificity of the cellular response to the heparin binding growth factor, including the proteins phosphorylated by tyrosine kinase activity.

BDNF exerts its effects on a wide variety of CNS cells including cortical neurons (Ghosh *et al.*, 1993), cholinergic neurons from the basal forebrain and hippocampus, and dopaminergic, serotonergic and GABAergic neurons from the ventral mesencephalon (Alderson *et al.*, 1990; Spenger *et al.*, 1995). These effects are initiated by the binding of BDNF (as well as all other neurotrophins such as NGF, NT-3, NT-4/5 and NT-6) to a common, relatively abundant low affinity receptor, and with respect to BDNF, specifically to the much more rare high affinity *trkB* receptor, followed by protein phosphorylation and retrograde transport of the factor and at least the low affinity receptor to the cell body (Thoenen *et al.*, 1987). It is this specific binding to the high affinity receptor which allows for the variety of effects observed with the neurotrophins. Several functions of the low affinity receptor, however, have been postulated including its

function as a 'presentation receptor' for the sequestration of the neurotrophin and its presentation to the high affinity receptor (Taniuchi *et al.*, 1988), its G-protein mediated signal mechanism (Feinstein and Larhammer, 1990), its involvement in the retrograde transport of NGF specifically (Johnson et al., 1987) or possibly its discriminatory identification potential with regards to specific neurotrophins (Rodriguez-Teber et al., 1992). Neurotrophin binding to the high affinity *trk* receptor seems, however, to be sufficient to elicit the biological response (Ibanez *et al.*, 1992) and therefore the relationship of the two forms of receptors remains unclear. The *trk* receptors (A, B and C) are a highly conserved receptors with intracellular tyrosine kinase domains and extracellular leucine, cysteine and immunoglobulin-like domains. These intracellular tyrosine kinase domains are distinct from those associated with the FGF receptors. The complexity of the distribution of neurotrophin receptors and factors, localization of tyrosine kinase activity and structural features that allow for appropriate binding of growth factors may lead to the specificity for neurotrophin actions (Wheeler and Bothwell, 1992). The inability to bind certain neurotrophins, most commonly NGF, has been shown in some systems to lead to programmed cell death or apoptosis (Oppenheim, 1985) which is morphologically distinct from injury- induced necrotic cell death. Apoptosis is characterized by nuclear and cytoplasmic condensation which break up into membrane-bound fragments and are readily phagocytosed by neighbouring cells. Transcriptional changes occur with increased expression of calmodulin, polyubiquitin, and *c-jun* and decreased

expression of *c-myc* and *c-myb* among others (Schwartzman and Cidlowski, 1993). Many hormone- and growth factor-responsive tissues including the thymus (Compton and Cidlowski, 1986), adrenal cortex (Wyllie *et al.*, 1973), embryonic cells (Rawson *et al.*, 1991) and neurons (Chang *et al.*, 1990; Rich and Hollowell, 1990) are subject to apoptosis upon removal or addition of the appropriate regulatory factor the process of which plays a major role in the regulation of cell populations.

The insulin-like growth factors (IGF-I and IGF-II) are ubiquitous throughout the body and exhibit effects resulting on cell proliferation, differentiation and metabolism (Heyner and Garside, 1994). Effects on neural development have been described, including modulation of neurotransmitter release and a regulation of cell proliferation (Sara and Carlsson-Skwirut, 1988). IGFs are synthesized as prohormones which are cleaved at specific points during their secretory phase. A truncated form of IGF-I which exerts potent neurotrophic effects in vitro has been described in the fetal and adult human brain (Sara et al., 1988; Carlsson-Skwirut *et al.*, 1986). These effects include stimulation of cell proliferation, DNA synthesis, oligodendrocyte development and myelination. The actions of IGF-I and IGF-II are mediated via a tetrameric receptor composed of 2 alpha and 2 beta subunits linked by disulfide bonds. The alpha subunit of the IGF-I receptor binds the factor extracellularly and the beta subunits traverses the membrane and contains the tyrosine kinase domain. IGF-II also binds to a much longer extracellular domain and short intracellular domain with no tyrosine kinase

activity; most of IGF-II's effects *in vitro* may result from binding to the IGF-I receptor (Giacobini *et al.*, 1990).

Tyrosine kinase activation by binding to growth factor receptors is critical for regulation of mitosis, cell growth and differentiation. Signalling pathways initiated by tyrosine kinases lead to nuclear events which eventually elicit dramatically different biological responses depending on the factor, environment and cell involved (Schlessinger and Ullrich, 1992). In some cases, ligand binding leads to receptor dimerization and therefore activation of the tyrosine kinase activity (Schlessinger, 1988) but also to autophosphorylation mediated by intracellular mechanisms (Lammers *et al.*, 1990). These tyrosine phosphorylated regions of growth factor receptors provide high affinity binding sites for many cellular signalling proteins possessing the src homology 2 domains (SH2)(Koch *et al.*, 1991; Heldin, 1991) resulting in specific and varied responses to growth factor interaction. BDNF binding to the trkB receptor, for example, in cortical glial cells leads to an increase in mitogen-activated protein (MAP) kinase tyrosine phosphorylation, MAP kinase activity, intracellular calcium concentration and *c-fos* (Roback *et al.*, 1995). Similarly, in rat hippocampal pyramidal cells, BDNF enhances the production of microtubule associated protein kinases and *c-fos* (Marsh *et al.*, 1993). BDNF has also been shown to modulate the release of another neurotrophin, NT-3, in rat cerebellar neurons (Leingartner *et al.*, 1994). EGF has been shown to elevate the activity of choline acetyltransferase through a mechanism mediated by glial cells in septal cholinergic neurons. In this same

system, bFGF acts similarly but through another mechanism not mediated by glia (Yokoyama *et al.*, 1994). EGF has also been shown to support the survival and proliferation of fetal rat mesencephalic precursor cells (Mytilineou *et al.*, 1992). Although growth factors have been shown exert a multitude of specific effects on target cells, only some of which, at this time, have been discovered, the physiological relevance is still uncertain.

1.2 Localization of Growth Factors and Receptor in the CNS

The brain has been shown to be one of the richest sources of aFGF and bFGF (Matsuyama *et al.*, 1992) and their respective mRNAs. FGFs exist mainly intracellularly or intranuclearly but few examples of extracellular FGF in the CNS have been described (Stopa *et al.*, 1990; Tooyama *et al.*, 1991). FGFs have been localized in both neurons and in glial cells but the cellular compartmentalization is a matter of controversy. Probably due to varying methods of tissue preparation and experimental protocols, results have not been consistent (Hanneken and Baird, 1992), although widespread localization has been described. bFGF gene expression has been demonstrated in the cingulate cortex and the CA2 region of the hippocampus (Emoto *et al.*, 1989) while nuclear and cytoplasmic localization has been shown in astrocytes and neurons of the hippocampus (Woodward *et al.*, 1992) as well as in Purkinje cells of the cerebellum (Matsuda *et al.*, 1992) and some dopaminergic neurons of the ventral mesencephalon (Tooyama *et al*, 1994).

In the adult brain, BDNF mRNA is found predominantly in the CNS and

particularly in hippocampal and cortical neurons (Maisonpierre *et al.*, 1990a) but detectable levels have not been demonstrated in the striatum or substantia nigra in the basal ganglia (Maisonpierre *et al.*, 1990b). BDNF mRNA has however been demonstrated in the developing embryonic rat brain (Maisonpierre *et al.*, 1990b) and the mRNA for the high affinity *trkB* receptor and BDNF suggest an expression by ventral mesencephalic dopaminergic neurons (Gall *et al.*, 1992).

Insulin-like growth factor II has been shown to be produced in the leptomeninges and the choroid plexus (Hynes *et al.*, 1988) however a specific site of IGF-I production in the brain has not been shown. Receptors for this growth factor, however, are widely distributed throughout the CNS with the greatest concentration found in the cortex and hippocampus during intrauterine growth (Lesniak *et al.*, 1988).

The system in which I have chosen to examine the effects of these growth factors is the population of dopaminergic neurons which project from the substantia nigra to the striatum forming the nigro-striatal pathway. This population of neurons is well delineated, abundant and easily accessible in the adult and fetal rat as well as the fetal human and is known to degenerate in Parkinson's disease (PD); clinical symptoms however do not appear until the loss of neurons reaches 80-90% of control. At this time, adequate long-lasting treatment for the disease is not available and it is not possible to halt or even slow the death of the dopaminergic neurons. Some of this dopaminergic cell death may occur from a loss of target-derived trophic support.

1.3 Striato-nigral Projection and Animal Models

Dopaminergic neurons of the substantia nigra pars compacta (the A9 cell group) project in a topographical fashion to various forebrain areas (Bjorklund and Lindvall, 1984) including a major projection to the striatum, and minor projections to the septum, nucleus accumbens and anteromedial and entorhinal cortices. This nigral projection is an integral component of the basal ganglia system which functions to modulate the initiation and intensity of movement to ensure a coherent and appropriate behavioural response (Herman and Abrous, 1994). The basal ganglia includes the interconnection of neuron projections linking the caudate and putamen to the substantia nigra, internal and external portions of the globus pallidus. Major interactions external to the basal ganglia involve the thalamus and the cortex where the integrated behavioural response is initiated. Lesion or stimulation of the dopaminergic projection to the striatum induces deficits in the corresponding target area (LeMoal and Simon, 1991).

Animal models used to study the nigro-striatal dopamine pathway include the injection of 6-hydroxydopamine (6-OHDA), a catecholaminergic neurotoxin, into the substantia nigra or medial forebrain bundle to deplete the target striatum of dopamine as well as other aforementioned forebrain sites. 6-OHDA is equestered by the high affinity dopamine uptake system into the catecholaminergic cells and exerts cytotoxic action through the production of superoxide radical species. 6-OHDA lesions in rat ventral mesencephalon are

usually limited to only one side and with the administration of dopamine agonists, typical rotational behaviour is demonstrated which presumably results from the imbalance in striatal dopamine receptor stimulation.

The compound, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), in its oxidized form, has emerged as a potent environmental agent which is responsible for the development of a parkinsonian-like syndrome in mice, primates and humans through ingestion, cutaneous contact or inhalation (Langston and Ballard, 1984). Individuals ingesting the toxin quickly develop a Parkinson-like syndrome which closely resembles Parkinson's disease in its motor aspects and responds identically to anti-parkinsonian drugs (Langston and Ballard, 1984). MPTP is a very lipophilic molecule that readily crosses the blood brain barrier and is taken up into CNS cells, most likely astrocytes, where it is oxidized by monoamine oxidase B to 1-methyl-4-phenylpyridinium (MPP+). MPP+ is selectively taken up into the SN dopaminergic neurons via the high affinity dopamine uptake system. Once in the cell, MPP+ attacks the mitochondria thereby exerting its action of selective cell death of these neurons.

It must be noted that the above mentioned models for this devastating disease cannot, however, mimic the characteristic slow progressive loss of dopaminergic neurons that is always seen in idiopathic PD.

1.4 Target Interactions During Development

Target cells of developing neurons in the CNS may play a particularly

important and specific role in development. For example, target cells in the striatum influence the survival but not differentiation of dopaminergic projection neurons in the substantia nigra (SN) (Dong *et al.*, 1993) as can hippocampal target cells influence cells in the basal forebrain (Dunnet *et al.*, 1986; Wainer *et al.*, 1991). Using sensory neurons *in vitro*, it has been shown that the timing of target interaction is critical, and varying responses to the same growth factors can result depending on the time of exposure (Vogel and Davies, 1991). Furthermore, such target effects can be complex and interactive.

The target cells may exert their effects on developing neurons via production of specific growth factors. Nerve growth factor has been shown to stimulate differentiation independent of survival by enhancing neurite outgrowth of sympathetic neurons *in vitro* (Thoenen *et al.*, 1971; Hefti *et al.*, 1982), and to prevent naturally occurring programmed cell death (Angeletti *et al.*, 1971). Target tissues have been shown to produce nerve growth factor (Heumann *et al.*, 1984), and projecting sympathetic neurons possess the appropriate receptors (Shelton and Reichardt, 1984) and to transport them anterogradely to the terminals where NGF binding occurs (Johnson *et al.*, 1987). Glial growth factors have been shown to influence the development of neural crest cells by redirecting their commitment from both neurons and glia to one of a strictly glial phenotype (Shah *et al.*, 1994). Fibroblast growth factors have been shown to regulate cell division of early neural progenitor cells (Gensburger *et al.*, 1987; Murphy *et al.*, 1990; Kilpatrick and Bartlett, 1993), and glial cell line-derived neurotrophic factor (GDNF) has been

shown to support the survival of midbrain dopaminergic neurons *in vivo* (Hudson *et al.*, 1995).

In this thesis, I have sought to explore some of the signals involved in epigenetic effects in the CNS at several of the stages described above. In particular, I have examined proliferative signals influencing embryonic progenitor cells, target interactions, especially those mediated by peptide trophic factors as well as the susceptibility of cells to toxic damage by a neurochemical metabolite.

With respect to proliferation, I have isolated progenitor cells from the human forebrain which divide in the presence of epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) which are active peptide growth factors in the central nervous system. Upon removal of these factors, the cells cease to divide, and differentiate into specific neurons and glia whose phenotypes I have characterized. Further characterization may however, lead to the production of pure phenotypic populations of cells possibly of a dopaminergic nature which could provide a large source of cells for cell replacement therapy for PD.

In order to explore the effects of target interactions on a neuronal population, I have studied the dopaminergic cells of the ventral mesencephalon because of their distinct brain localization and accessability in both the adult and the embryo. These cells are of clinical importance in the context of Parkinson's disease where there is a profound decrease in the number of dopaminergic

neurons of the substantia nigra. The target striatum is known to exert trophic effects on dopaminergic cells *in vitro*. When grown in the presence of striatal extract from animals whose SN had been chemically or mechanically lesioned, the survival and growth of dopaminergic neurons is enhanced (Tomozawa and Appel, 1986; Carvey *et al.*, 1989; Prochiantz *et al.*, 1991). Lesioning the target area has allowed me to examine the retrograde consequences within the substantia nigra. Lundberg *et al.* (1994) have shown in similar experiments that target lesioning leads to a decrease in cell size but no change in cell number in the substantia nigra pars compacta (SNc) although others have demonstrated a cell loss (Forno *et al.*, 1983; Pasinetti *et al*, 1991). I hypothesized that the target striatal cells provide a trophic influence for the projection neurons from the substantia nigra, and that destroying this target would lead to a degeneration of SN neurons. For this purpose, it is important to distinguish retrograde death due to axonal damage from that due to loss of trophic support. This I have done by replacing the lost trophic support in the form of fetal grafts.

1.5 Growth Factor Effects on Dopaminergic Neurons

Having demonstrated that the target cells provide a trophic influence to the projection neurons of the SN, the identification of these factors becomes of great interest because of the clinical relevance of this group of cells. Neurotrophic factor effects include enhancement in growth, survival, differentiation and maintenance of neurons. Brain-derived neurotrophic factor (BDNF), insulin-like

growth factor I (IGF-I) and basic fibroblast growth factor (bFGF) have all been shown to exert trophic influences on rat ventral mesencephalic dopaminergic neurons *in vitro* (Knusel *et al.*, 1990; Hyman *et al.*, 1991; Beck *et al.*, 1993; Beck, 1994). Recently, GDNF has been shown to exert similar effects *in vivo* (Lan *et al.*, 1993; Hoffer *et al.*, 1994; Bowenkamp *et al.*, 1995). The effects of growth factors on *human* dopaminergic neurons has not, however, been adequately studied. Spenger *et al.* (1995) have shown increased tyrosine hydroxylase cell density in human ventral mesencephalic dopaminergic neurons after exposure to BDNF *in vitro* with no examination of cell survival. Silani *et al.* (1994) have shown similar effects with bFGF and that these effects were mediated by glia. Here I confirm that BDNF and bFGF enhance human dopaminergic cell survival *in vitro* and show that the greatest effect is seen when bFGF is delivered to the cells together with heparin. Heparin has been suggested to allow more efficient binding of the growth factor to the cells (Fuxe *et al.*, 1994).

1.6 Treatments for Parkinson's Disease

The most common pharmacological treatment of PD involves drug therapy with dopaminergic agonists to mimic the effects of dopamine, anticholinergics to balance the neurotransmitter levels in the striatum, and monoamine oxidase inhibitors to reduce the catabolism of dopamine (LeWitt; 1991), however, drug therapy rarely alleviates a majority of parkinsonian symptoms over time. When drug therapy has been ineffective or inadequate, stereotactic surgery can be

performed in which portions of the basal ganglia are lesioned in an attempt to balance the neuronal activity. Stereotactic surgery targets rigidity and tremor symptoms while an effect on akinesia has not been described (Narabayashi, 1990) and therefore rigid clinical assessment is imperative prior to any surgical attempts. Pallidotomy, which involves the identification of tremor associated loci in the globus pallidus followed by thermocoagulation of the identified area results in specific lesions of these symptom responsive areas. This procedure is performed on an awake patient and therefore lesion effects are immediately evident and maximal correction is achieved (Narabayashi, 1990).

Alternatively, dopaminergic neuron replacement has been attempted experimentally, with grafts into the deafferented target of the dopaminergic ventral mesencephalic cells. The success of grafting experiments in both rodent (Björklund and Stenevi, 1979; Perlow *et al.*, 1979) and monkey (Bakay *et al.*, 1987; Bankiewicz *et al.*, 1988; Redmond *et al.*, 1986; Fine *et al.*, 1988; Sladek *et al.*, 1988) and perhaps more importantly the success of grafting human VM cells into the deafferented, immunosuppressed rat striatum (Stromberg *et al.*, 1986; Brundin *et al.*, 1988) has led to the extension of this protocol to a treatment for PD. Fetal human ventral mesencephalic dopaminergic neurons have been grafted into the caudate and/or putamen of patients with idiopathic PD (Lindvall *et al.*, 1988; Madrazo *et al.*, 1988; Hitchcock *et al.*, 1988; Molina *et al.*, 1991; Freed *et al.*, 1990; Redmond *et al.*, 1989; Spencer *et al.*, 1992; Fine *et al.*, 1993). Groups performing transplantation procedures in patients with idiopathic PD and in those

individuals with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism have reported varying success, from modest benefit to little or no improvement (Freed *et al.*, 1992; Widner *et al.*, 1992; Lindvall *et al.*, 1994; Freeman *et al.*, 1994; Peschanski *et al.*, 1994; Spencer *et al.*, 1992; Henderson *et al.*, 1992). Possible explanations for this limited success compared with animal models of the disease include reduced graft survival, perhaps because the grafted cells may be affected by the progression of the disease or by a yet unidentified causative agent present in the diseased CNS affecting all dopaminergic neurons. An obvious difference between grafting fetal cells into the striatum of experimentally lesioned animals as compared to patients with idiopathic PD is the fact that for years prior to the transplantation, virtually all PD patients receive daily dopamine replacement therapy with L-DOPA. In almost all rodent and primate transplantation experiments, no such treatment was given. Prolonged L-DOPA administration to 6-hydroxydopamine-lesioned rats exerts profound effects on dopamine receptors, which become upregulated in the striatum due to the loss of dopaminergic input from the SN (Heikkila *et al.*, 1981). It is therefore important to consider that L-DOPA may exert toxic effects on the grafted fetal dopaminergic neurons directly. I have therefore examined the susceptibility of both rat and human dopaminergic cells to toxic damage by the neurochemical precursor of dopamine, L-DOPA.

2.0 Propagation and Inducible Differentiation of Human Forebrain Neural Progenitor Cells

2.1 Introduction

During development, neuroepithelial cells of the embryonic neural tube proliferate and differentiate into the diverse neurons and glia of the mammalian central nervous system (CNS). Multipotential progenitor cells derived from fetal rodent striatum (Temple, 1989), cerebral cortex (Williams *et al.*, 1991; Davis and Temple, 1994), hippocampus (Ray *et al.*, 1993), midbrain (Kilpatrick and Bartlett, 1993), spinal cord (Ray and Gage, 1994) and adult striatum (Reynolds *et al.*, 1992) have been grown *in vitro* and found capable of generating neurons and glia. Propagation of similar progenitor cells of human origin, however, has not yet been described.

The regulation of this proliferation and differentiation is not well understood, but a number of factors influencing the fate of rodent CNS progenitor cells have been identified. Basic fibroblast growth factor (bFGF) can stimulate proliferation of multipotential progenitor cells from fetal rodent telencephalon, mesencephalon (Kilpatrick and Bartlett, 1993; Vescovi *et al.*, 1993) and spinal cord (Ray and Gage 1994) and can regulate the proliferation of oligodendrocytes (McKinnon *et al.*, 1990; Gard and Pfeiffer, 1993; Deloulme *et al.*, 1992; Bögler *et al.*, 1990; Mayer *et al.*, 1993). Epidermal growth factor (EGF) exerts a mitogenic action on fetal rodent CNS cells, including glia (Westermark, 1976; Leutz and

Schachner, 1981); EGF can also act on multipotent progenitor cells yielding mixed cultures of neurons and astrocytes (Reynolds and Weiss, 1992; Reynolds *et al.*, 1992). Neurotrophic effects of EGF, including the enhancement of survival and neurite outgrowth of postnatal rat striatal, cortical and cerebellar neurons in primary culture, have also been demonstrated (Knusel *et al.*, 1990; Morrison *et al.*, 1987; Morrison *et al.*, 1988). Insulin-like growth factors (IGF-I and IGF-II) have also been found to be growth promoting hormones (Sara and Carlsson-Skwirut, 1988). *In vitro* studies have demonstrated effects of IGF-I on neurons, glia and chromaffin cells, including enhancement of cell survival, DNA synthesis, proliferation, oligodendrocyte development and myelination (McMorris *et al.*, 1986; Sara and Carlsson-Skwirut, 1990; Drago *et al.*, 1991; Frödin and Gammeltoft, 1994).

Here I report that EGF and IGF-I, when added to a partially defined medium, allow the survival and proliferation of progenitor cells from fetal human forebrain, including primordia of the cortex, striatum and basal forebrain. These progenitors can be maintained in a proliferating state for months in partially defined medium supplemented with EGF and IGF-I; at any time, they can be induced to differentiate by supplying an appropriate substrate for attachment and replacing the partially defined medium with one containing high serum and no added growth factors into neurochemically defined neurons and glia consistent with the cell types found in these forebrain areas in the mature state.

2.2 Materials and Methods

Human fetal brain tissue was obtained from routine therapeutic abortions performed at the Victoria General Hospital in Halifax, Nova Scotia, with informed consent and approval of appropriate institutional ethical review boards. Evacuated tissue fragments from 6-8 week post-conception fetuses, as staged according to the developmental atlas of England (1983), were collected directly into sterile ice-cold isotonic saline with heparin (10μg/ml). Ventral forebrain (excluding dorsal forebrain in all cases but including ventral telencephalic and diencephalic structures such as striatal eminences and basal forebrain) was dissected under stereomicroscopic observation in a laminar flow containment hood, and transferred to a solution of 0.05% (w/v) trypsin (Type XIII, Sigma) in calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS) for 20 minutes at 37°C. This incubation was followed by 4 washes with 0.01% deoxyribonuclease (DNase I, Sigma) in complete HBSS. The tissue was then dissociated to a suspension of single cells in 0.01% DNase I in CMF-HBSS by repeated gentle passage through fire-polished Pasteur pipettes of decreasing bore size. Viability of the dissociated cells was assessed by exclusion of ethidium bromide, after incubation in a solution of acridine orange and ethidium bromide (1μg/ml each in HBSS). Cells were counted in a haemacytometer using standard fluorescein and rhodamine fluorescence filters to detect acridine orange- and ethidium bromide-containing cells, respectively. Cells were plated at $2x10^3$ viable cells per mm^2 in 24-well uncoated Nunc tissue culture dishes. Progenitor cell

culture medium (PCM) was composed of a 3:1 mixture of Dulbecco's modified Eagle medium (DMEM; Gibco) and Ham's F-12 (Gibco), with 5%(v/v) horse serum (Gibco), insulin (Sigma, 10 μg/ml), transferrin (Sigma, 200μg/ml, progesterone (Sigma, 40 nM), putrescine (Sigma, 200 μM) and sodium selenite (Sigma, 60nM). This PCM was further supplemented with either EGF (Upstate Biochemicals, 20ng/ml) (PCM-E) or EGF plus IGF-I (Upstate Biochemicals, 100ng/ml) (PCM-EI). Cells were fed at 3-4 day intervals throughout the experiment with PCM, PCM-E or PCM-EI. At intervals of 30-60 days *in vitro* (DIV), resulting spherical cell masses were dissociated as above, and cells replated at a density of $2x10^3$ viable cells per mm^2.

To induce differentiation, spherical proliferating cell masses were dissociated as above, and the resulting cell suspension plated onto poly-L-lysine (Sigma)-coated dishes in DMEM with 10% fetal bovine serum (FBS). For cell cycle analysis, spherical masses of proliferating cells were, while still in PCM, exposed to 1 μg/ml bromodeoxyuridine (BrdU; Sigma), which is incorporated into the nuclear DNA of proliferating cells, for 6, 12, 18, 24, 36, 48, 60 or 72 hours. Cell masses were then dissociated, and the resulting cell suspension plated onto poly-L-lysine-coated plates in DMEM with 10% FBS. After 12 hours, these cells were fixed with 4% paraformaldehyde in 0.1M phosphate buffer, Ph 7.4, for 20 minutes at room temperature. For phenotypic analysis of progenitor cell progeny, some cells exposed to BrdU for 48 hours were grown on coated plates in DMEM with 10% FBS for up to ten days before fixation and staining.

Immunocytochemical staining was carried out as previously described (Chalmers and Fine, 1991; Fine, 1993), using well-characterised antibodies specific for BrdU (Amersham) to identify cells which divide in culture, neurofilament 200 (NF; Sigma) to identify neurons, glial fibrillary acidic protein (GFAP; Sigma and DAKO) which binds proteins characteristic of astrocytes, a variety of specific neuronal phenotype markers including serotonin (5-HT; Eugene Tech), choline acetyltransferase (ChAT; Chemicon), glutamic acid decarboxylase (GAD; Calbiochem), tyrosine hydroxylase (TH; Eugene Tech), dynorphin (Penninsula Laboratories, Inc.), substance P (Sub-P; Eugene Tech) and aromatic amino acid decarboxylase (AADC; Eugene Tech), an enzyme present in catecholaminergic neurons . Other antibodies were used including those directed to galactocerebroside (GalC; Boehringer Mannheim) to identify oligodenroglia, human nerve growth factor receptor (NGF-R; American Type Culture Collection) which has been shown to be colocalized with choline acetyltransferase in cholinergic neurons, A2B5 (Boehringer Mannheim) which binds a tetrasialoganglioside common to neurons and type 2 astrocytes and the neuronal/glial lineage marker (Raff et al., 1983), nestin-129 (Tohyama et al., 1992; kindly supplied by R. McKay, NIH). Also, cells were incubated with FITC-labelled tetanus toxin (FITC-TT; List Biologicals Laboratories, Inc) which binds specifically to a polysialoganglioside on the surface of neurons. Cells were fixed for 20 minutes at room temperature by replacing culture medium with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4. Cells to be stained for

BrdU were incubated with 0.1% (w/v) DNase in 0.01M phosphate buffer prior to primary antibody treatment, to increase antibody access to incorporated BrdU. Dilutions of primary antibodies or TT in 0.1M phosphate buffer containing 1% normal serum were as follows: rabbit anti-GFAP (1:100), rabbit anti-NF (1:200), mouse anti-BrdU (neat), rabbit anti-5-HT (1:1000), rabbit anti-ChAT (1:1000), rabbit anti-GAD (1:200), mouse anti-GalC (1:200), mouse anti-NGF-R (neat), rabbit anti-TH (1:1000), rabbit anti-Sub-P (1:1000), mouse anti-A2B5 (1:100), rabbit anti-nestin-129 (1:2000), rabbit anti-AADC (1:5), rabbit anti-dynorphin (1:1) and FITC-TT (200μg/ml). Bound antibodies were localized by immunofluorescence, using Texas Red-conjugated sheep anti-mouse (Amersham) and fluorescein-conjugated donkey anti-rabbit (Amersham) immunoglobulins diluted 1:30. Nestin-IR was localized using diaminobenzidine (DAB) as follows. After nestin primary antibody incubation, the spheres were washed 4 times with PBS and incubated for 1 hour at room temperature with a biotinylated goat anti rabbit IgG (1:250) followed by a 1 hour incubation with avidin-biotin-peroxidase complex (0.6% v/v) at room temperature. Peroxidase was reacted with DAB (0.05% w/v) and hydrogen peroxide (0.003% v/v) to produce a brown reaction product. To determine the proportion of cells incorporating BrdU, all BrdU-labelled and unlabelled cells were counted in 10 randomly-selected fields of view in each well using a 40x objective (n=6) with epifluorescence and differential interference-contrast optics. BrdU-labelled cells were expressed as percent of total cells. Stained cells were imaged by epifluorescence and recorded on photographic film or digital CCD

camera. Fluorescent filters sets used were Zeiss 510-560, FT 580, LP 590 and 450-490, FT 510, 515-565 to view broad spectrum red and green fluorescence.

2.3 Results

Human fetal ventral forebrain tissue, including primordia of the cortex, striatum and basal forebrain, plated in PCM-E at a density of $2x10^3$ cells per mm^2 on uncoated tissue culture plastic consistently formed spherical cellular masses 0.2-1.5mm in diameter after 30-60 DIV (Fig. 1A). This mass formation was not seen with cells plated at higher density ($>5x10^3$ cells per mm^2) on uncoated tissue culture plastic, nor with cells plated on poly-L-lysine-coated dishes at densities of $\leq 2x10^3$. Cells grown in PCM without additional additives did not produce growth of these masses (Fig. 1B), while theformation of these masses was enhanced in PCM-EI (Fig. 1C). Thus for all further experiments, cells were grown in PCM-EI.

Immunohistochemical staining revealed nestin-immunoreactive cells within the cellular masses when these masses were plated, undissociated, directly onto poly-L-lysine-coated plates for 48 hours (Fig. 2). The masses attached loosely to the plate during this time period via small numbers of processes. After dissociating and replating the cells of these masses, 'secondary' cell masses formed; growth of secondary cells masses was less rapid than that of primary cell masses. After a further 30-60 DIV, secondary masses were similarly dissociated to produce 'tertiary' masses of proliferating cells. Such passage could be repeated at

Figure 1. The appearance of human fetal forebrain-derived cells after 4 weeks *in vitro*. A. In the presence of PCM supplemented with EGF, spherical cell masses begin to form. (Scale bar measures 75μm) B. In unsupplemented PCM, small cell masses may form by aggregation, but do not proliferate. (Scale bar measures 75μm) C. PCM supplemented with EGF and IGF-I leads to enhanced cell mass formation. (Scale bar measures 140μm) Differential interference contrast optics.

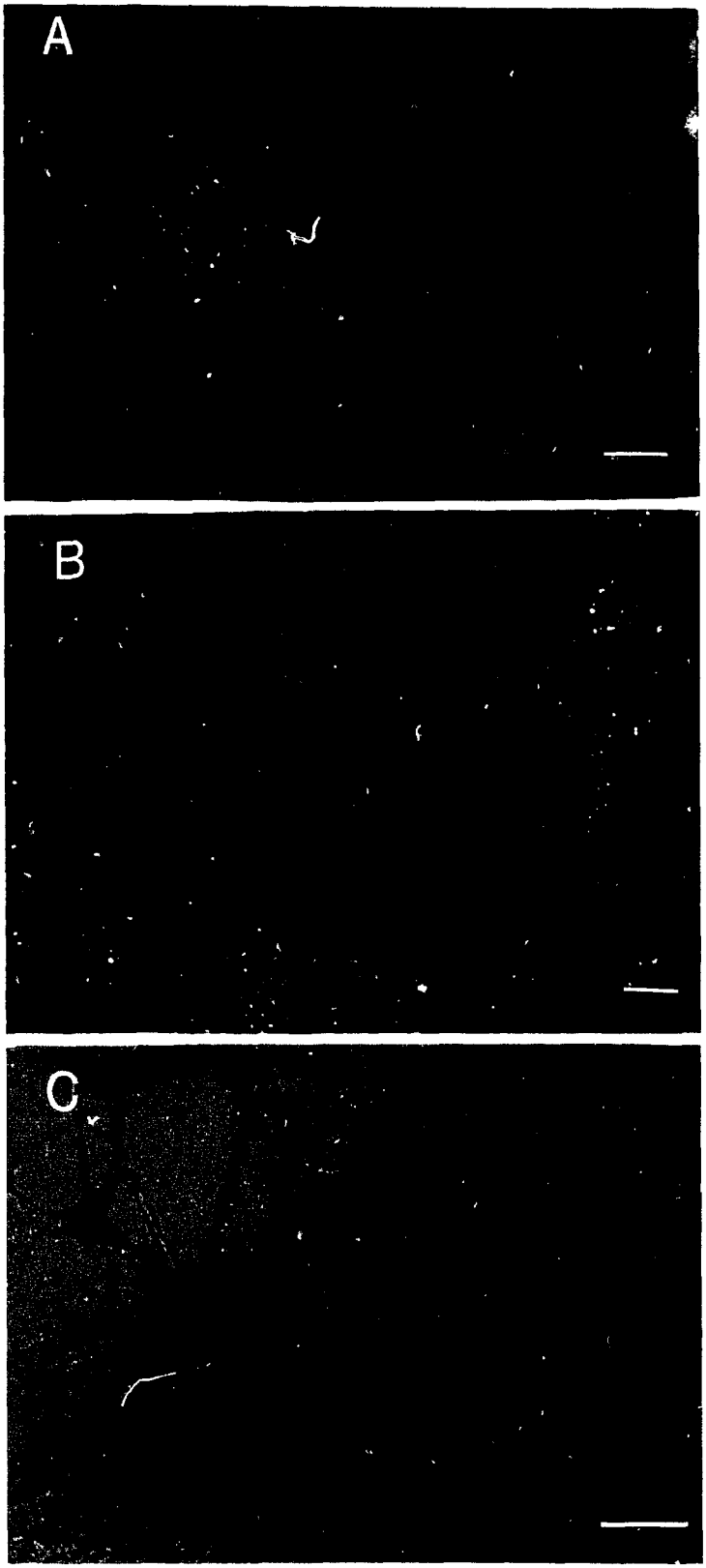


Figure 1.

Figure 2. Nestin immunoreactivity (arrows) within an undissociated mass of CNS progenitor cells 2 days after transfer to a poly-L-lysine coated dish. Arrowheads indicate unstained cells. (Scale bar measures 30μm)

Figure 2

least 2 times.

Tertiary cell masses, collected at least 75 days after initial primary plating to ensure that neuronal phenotypes resulted from cell division *in vitro* and not from differentiated neurons present in the initial plating, were re-dissociated and plated as single cells onto poly-L-lysine coated tissue culture dishes. After up to ten DIV, these cells were fixed and stained immunohistochemically to evaluate their cytochemical phenotype (Fig. 3); cells immunoreactive for ChAT (Fig. 3A), TH (Fig. 3B), TT (Fig. 3C), NF (Fig. 3D), Sub P (Fig. 3E), GAD (Fig. 3F), GFAP (Fig. 3G) and A2B5 (Fig. 3H) were evident. No 5-HT-, GalC-, NGF-R-, AADC- or dynorphin-immunoreactivity was detected although culture conditions may not have been optimal for the expression of these phenotypes. When these cells were derived from cellular masses incubated with BrdU for 48 hours immediately prior to dissociation, cells of several neurochemical phenotypes were found with BrdU-labelled nuclei (Figs. 3D-G).

To establish the fraction of cells actively replicating, tertiary cell masses were incubated with BrdU for various periods immediately prior to dissociation and replating; plated cells were fixed and stained immunocytochemically to detect BrdU-labelled cells. The percentage of cells incorporating BrdU into their nuclei increased with increasing incubation times from 12 to 36 hours; with longer incubations, the proportion of labelled nuclei remained unchanged at approximately 80% of the total (Fig. 4).

Figure 3. Fluorescence immunohistochemical staining of differentiated cells derived from CNS progenitor cells. Cells were grown *in vitro* for up to ten days in DMEM with 10% fetal bovine serum in poly-L-lysine-coated wells before fixation with 4% paraformaldehyde and subsequent staining for the neurochemical markers. A, choline acetyltransferase; B, tyrosine hydroxylase; C, tetanus toxin; D, neurofilament 200 protein (green); E, Substance-P (green); F, glutamic acid decarboxylase (green); G, the glial marker, GFAP (green) and H, the glial-neuronal lineage marker A2B5. Double staining with Texas Red labelled anti-BrdU was performed on specimens D-G; BrdU labelled nuclei indicate cells born *in vitro* after BrdU incorporation. (Scale bars measure 25μm)

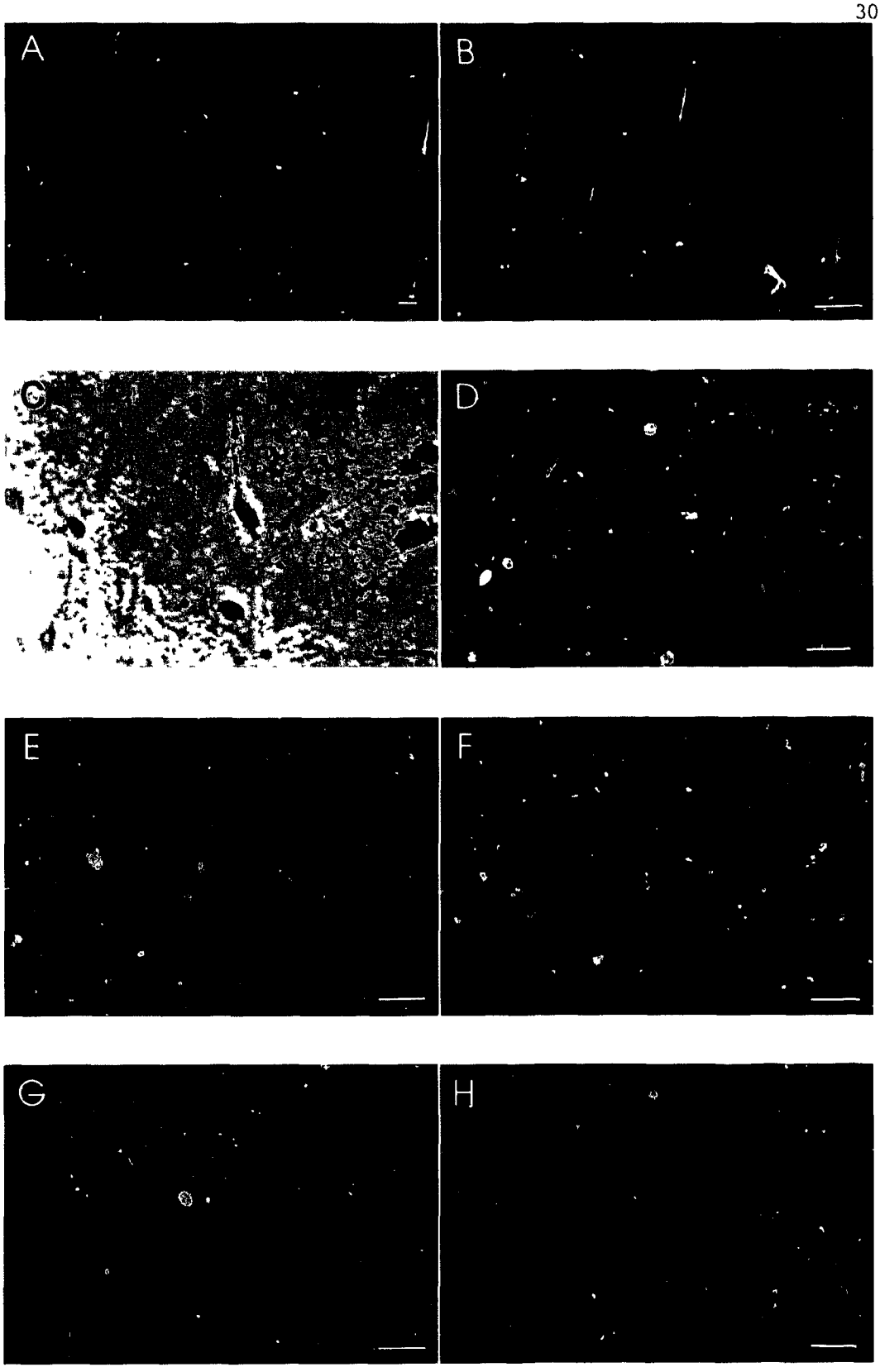


Figure 3.

Figure 4. Points indicate the fraction of mitotic cells as revealed by the presence of BrdU-IR nuclei after increased time of exposure to BrdU. IGF-I and EGF induced cell division in progenitor cell masses propagated *in vitro*. Vertical lines indicate S.E.M.

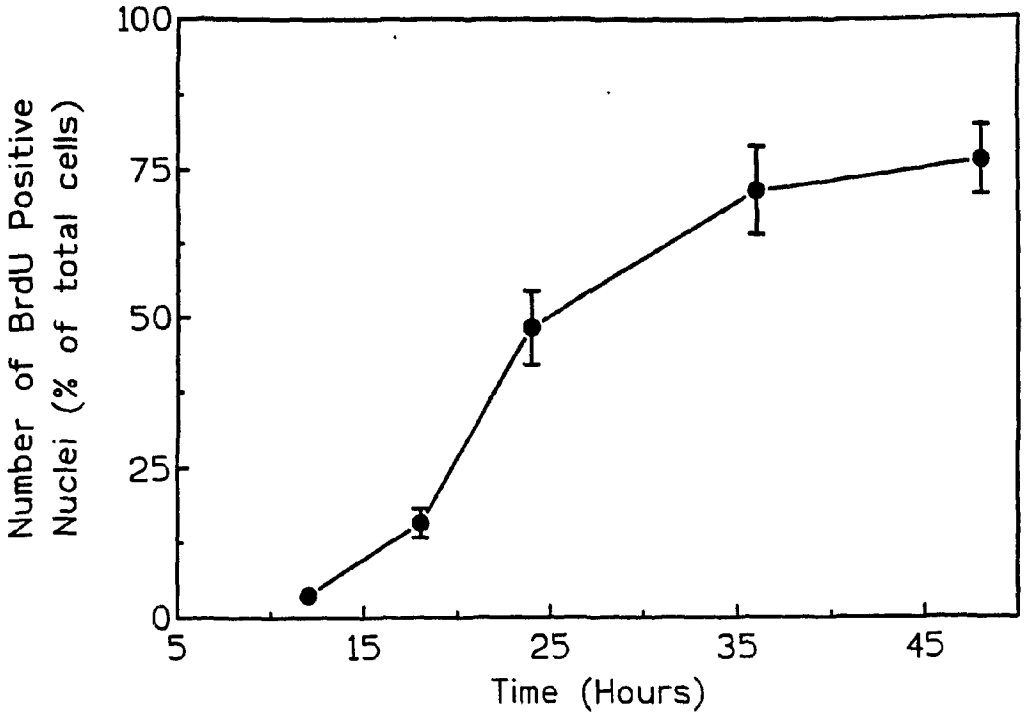

Number of BrdU Positive Nuclei (% of total cells)
100
75
50
25
0
5
15
25
35
45
Time (Hours)

Figure 4.

2.4 Discussion

These results demonstrate that EGF and IGF-I, when added to a partially defined medium, permit the survival and continued proliferation *in vitro* of progenitor cells from the fetal human forebrain for at least 4 months. Such cultured progenitor cells remain in an undifferentiated state, expressing nestin, until they are stimulated to differentiate. Here, differentiation was induced by changing culture conditions from a partially defined, low serum to high FBS-supplemented medium in culture dishes coated to promote attachment. A wide range of differentiated cells can in this way be produced, including neurons (as indicated by expression of neurofilament and tetanus toxin binding) of several neurochemical classes (catecholaminergic as indicated by TH, peptidergic as indicated by Sub-P, cholinergic as indicated by ChAT and GABAergic as indicated by GAD expression) and astrocytes (as indicated by GFAP expression). These differentiated neurons can be maintained *in vitro* for at least 15 weeks when cultured on glial feeder layers. All these differentiated cell types derived from progenitor cells of forebrain origin are also present in the normal adult forebrain. Lack of 5-HT staining was not surprising due to the absence of this neuronal phenotype in the adult ventral forebrain however, GalC, NGF-R, AADC and dynorphin are normally expressed. Inappropriate culture conditions and normal temporal appearance of oligodendrocytes may provide explanations for the lack of GalC staining. Oligodendrocytes have not been identified prenatally in the rat CNS although this oligodentrocyte 'birthday' has not been described in the human

fetus. Interestingly, a previously-described EGF-supplemented defined culture medium (Reynolds and Weiss, 1992) did not induce the formation of progenitor cells from either the forebrain or the ventral mesencephalon of human fetal origin.

To rule out the possibility that the histochemically identified cells were differentiated primary cells that had somehow persisted in culture, rather than differentiated offspring of progenitor cells replicating *in vitro*, progenitor cells were incubated with BrdU at a concentration that would be incorporated into the DNA but not affect proliferation. The cells were then induced to differentiate, and stained for both BrdU and particular neurochemical markers. Double-labelled cells, immunoreactive for BrdU and for substance P, neurofilament or GAD were observed, establishing that all of these cells could be produced by progenitor cells replicating in culture prior to terminal differentiation. Double-labelled astrocytes (immunoreactive both for BrdU and for GFAP) were also routinely observed; however, as these differentiated cells retain the ability to replicate, it is possible that astrocytes were derived from differentiated primary cells rather than from progenitor cells. This possibility could be tested by analysis of clones derived from individual progenitor cells. However, proliferation of these human forebrain progenitor cells appears to require a minimum cell density, and we have as yet been unable to obtain such clonal growth.

Cell cycle analysis (Fig. 4) indicates that the progenitor cells proliferate with a cell cycle less than 48 hours, and that approximately 75% of the clustered cells are mitotically active. Mitotic division of true stem cells would yield one

committed cell and another stem cell; alternatively, division of uncommitted progenitor cells could yield two other progenitor cells. The latter would yield a large proportion of proliferating cells while the former only a small fraction of the total over the time frame described here. The data of Fig. 4 suggest that the cells described here are predominantly progenitors with a subpopulation of stem cells, since even after many weeks in culture, the majority of cells are still undergoing mitosis. Progenitor cells can be shown to exhibit differentiation potential as well as a capacity for asymmetric cell division (Hall and Watt, 1989), which has been demonstrated in this preparation. Cells unlabelled with BrdU during the 48 hour incubation period have presumably ceased proliferation for unknown reasons which may include diffusive constraints, spontaneous differentiation, effects of the BrdU itself or may result from the division of potential stem cells into similar stem cells and committed cells although this has not been examined as of this time.

Trophic factor support for the proliferation and differentiation of neuronal cells has been demonstrated by others (Cattaneo and McKay, 1990; Gensburger *et al.*, 1987; Almazan *et al.*, 1985). The ability of EGF and IGF-I to support proliferation of these undifferentiated progenitor cells supports the possibility that these mitogenic growth factors play a role in normal development of the fetal human forebrain. IGF-I mRNA has been shown to be present in the human fetal CNS (Han *et al.*, 1988) and similarly EGF receptors have been localized throughout the adult human brain (Werner *et al.*, 1988).

Primary human fetal brain tissue may be useful for diverse purposes

including neural transplantation, pharmaceutical testing and studies of neural growth and differentiation; such uses, however, are constrained by practical and ethical considerations. The establishment of means for propagation and differentiation of human CNS progenitor cells, described here, should permit the production of large numbers of developing human neurons and glia for these and other purposes. Factors influencing the direction of their differentiation, leading to preferential production of one rather than another neurochemical phenotype, are currently under investigation. These cells may be useful for pharmaceutical and toxicological research, for clinical and laboratory transplantation with or without prior genetic engineering, and for other purposes.

3.0 Influence of Striatal Target Cells on Dopaminergic Neurons *in Vivo*: Lesion and Transplant Studies

3.1 Introduction

Environmental signals including factors derived from a variety of target tissues can exert profound effects on the development and survival of projection neurons in the CNS (Dal Toso *et al.*, 1988; Tomozawa and Appel, 1986; Prochiantz *et al.*, 1979; Friedman *et al.*, 1988). Retrograde changes have been observed in CNS nuclei following axotomy or destruction of target tissues (Tuszynski *et al.*, 1990). The nature of these changes remains controversial, however, as decreases in number (Fisher and Björklund, 1990; Pasinetti *et al.*, 1991; Forno 1983; Tamura *et al.*, 1990) as well as size (Lundberg *et al.*, 1994; Sofroniew *et al.*, 1990; Sofroniew *et al.*, 1985) of projection neurons have been reported. Numerous studies of the dopaminergic projection from the substantia nigra pars compacta (SNc) to the striatum have shown retrograde cell loss in the SNc following axotomy of the medial forebrain bundle or excitotoxic lesion of the striatum (Lapchak *et al.*, 1993; Pearson *et al.*, 1987; Pasinetti., 1991; Ichitani *et al.*, 1991; Krammer, 1980); however, a decrease in cell size without a decrease in cell number has also been reported (Lundberg *et al.*, 1994).

I undertook to clarify the effects of target striatal cells in the adult rat striatum on the number and size of the projection neurons from the SNc. Because disappearance of neurochemical staining, in this case tyrosine

hydroxylase, does not necessarily indicate cell death (Lams *et al.*, 1988), I performed prior retrograde labelling with fluorescent latex beads to distinguish dead cells from SNc cells that survived but no longer expressed the catacholaminergic marker, tyrosine hydroxylase (TH). The absence of TH-IR may indicate that the cells have entered a death directed cycle or simply a quiescent phase from which it is possible to manipulate them back to normal dopaminergic neuron activity by replacement of growth factors and functional connections in the form of target grafts. These grafts may also provide the necessary substrate for normal synaptic connections and therefore activity of these dopaminergic cells leading to more normal activity of the previously unlabelled cells. In this study, I report that ibotenic acid lesioning of the striatum leads to increased numbers of retrogradely labelled ipsilateral SNc cells that do not express TH. Furthermore, grafts of fetal striatal primordia to the lesioned striatum reduce lesion-associated cell death after 6 weeks.

3.2 Materials and Methods

Young adult female Sprague-Dawley rats (Charles River, Montreal, Canada) weighing 175-200 g at the beginning of the experiment were divided into 4 groups (Fig 5). Group A consisted of 9 rats receiving unilateral ibotenic acid lesions in the caudate-putamen. Group B consisted of 8 animals receiving bilateral intrastriatal injections with fluorescent retrogradely transportable microspheres for retrograde transport; 3 days later, the caudate-putamen on one

side was lesioned by ibotenic acid injection. The 6 rats in group C received ipsilateral intrastriatal grafts of fetal striatal primordia approximately 2 weeks after unilateral ibotenic acid lesions. Group D represented a naive untreated control group of 6 animals which did not receive lesions, microsphere injection or grafts. Six weeks after the lesioning of all animals (Fig 5), they were deeply anaesthetized and killed by transcardial perfusion with paraformaldehyde (PFA). All brains were subsequently removed and sectioned through the striatum and substantia nigra. Sections were then stained immunohistochemically to reveal tyrosine hydroxylase-immunoreactive (TH-IR) cells. Control rats in Group D were killed at the same developmental times as in all other groups. All surgery was performed under sodium pentobarbital anaesthesia (65mg/kg body weight i.p.). A total of 2μl of ibotenic acid (Sigma), at a concentration of 20μg/μl in 0.1 M phosphate buffer, pH 7.4, was injected unilaterally via a 30 gauge needle over 4 minutes into the striatum at four injection sites: (1) A = 0.4; L = 3.9; V = 5.3; (2) A = 0.4; L = 3.1; V = 4.0; (3) A = 1.3; L = 3.1; V = 4.4; (4) A = 1.3; L = 2.0; V = 4.3 (A = anterior from bregma; L = right of midline; V = ventral to dura; all coordinates are in millimetres, with toothbar level with the interaural line). Red fluorescent latex microsphere (Lumafluor, New City, NY) injections of 0.1 μl each were delivered bilaterally into the striatum at the same coordinates 3 days prior to unilateral lesioning using a 10 μl Hamilton syringe with a pulled glass capillary tube needle. Six weeks after ibotenic acid lesioning, all animals were perfused transcardially with 4% paraformaldehyde (PFA). Brains were removed and post

Figure 5. Diagrammatic representation of the course of treatment for the examination of target effects on projection neurons. Group A (n=9) received a unilateral ibotenic acid (IA) lesion in the striatum. Group B (n=8), prior to similar lesioning, received bilateral fluorescent bead injections to the same striatal coordinates. Animals in group C (n=6), after unilateral ibotenic acid lesioning, received an ipsilateral fetal striatal graft. Group D (n=6) represents an untreated control group.

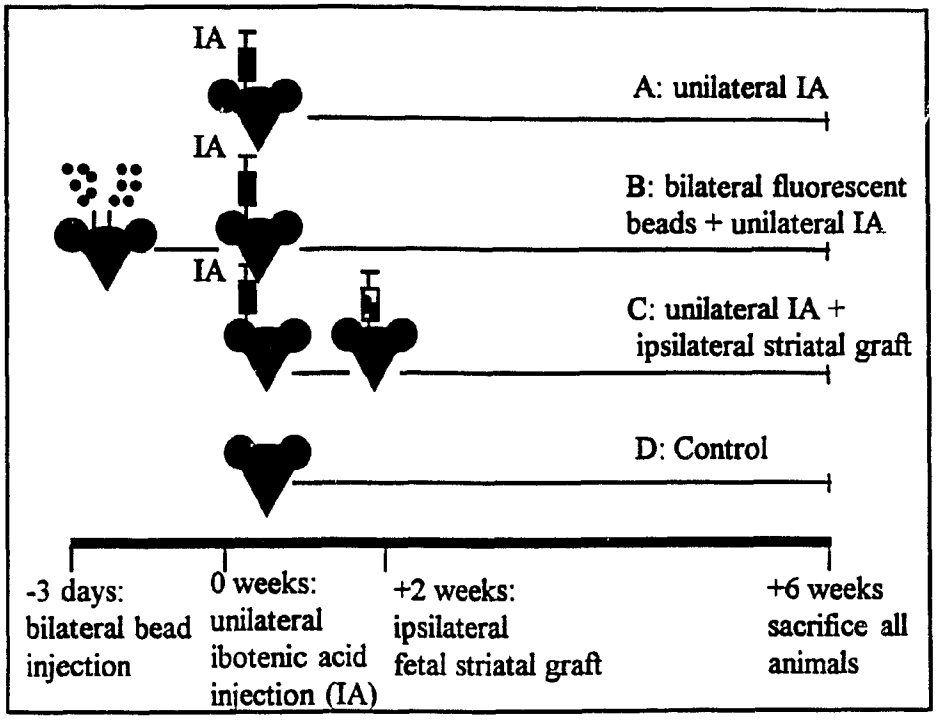

IA
A: unilateral IA
IA
B: bilateral fluorescent beads + unilateral IA
IA
C: unilateral IA + ipsilateral striatal graft
D: Control
-3 days: bilateral bead injection
0 weeks: unilateral ibotenic acid injection (IA)
+2 weeks: ipsilateral fetal striatal graft
+6 weeks sacrifice all animals

Figure 5

-fixed with 4% PFA for 24 hours at 4°C.

Group C animals, 2 weeks after lesioning, received ipsilateral intrastriatal grafts of fetal striatal eminence cells from 16-17 day rat embryos. Embryonic striatal eminence was dissected, and incubated for 20 min at 37°C in 0.05% (w/v) trypsin (Type XIII, Sigma) followed by four washes in ice-cold 0.01% (w/v) deoxyribonuclease (DNase I) (Sigma). Both enzymes were dissolved in calcium- and magnesium-free balanced salt solution containing 0.6% (w/v) D-glucose. The embryonic striatal eminence fragments were then dissociated in DNase solution by gentle pipetting with decreasing diameter bore sizes. The cells were suspended to a density of 250,000 cells/μl, and 1μl was injected at coordinates previously injected with ibotenic acid. Cells were delivered to the graft site using a 10μl syringe (needle o.d. 250μm, i.d. 150μm). Six weeks after transplantation, these rats were perfused transcardially with 4% PFA. Brains were removed and post-fixed in 4% PFA for 24 hours at 4°C.

Sections from animal in groups A, C and D were processed for TH-IR (Chalmers and Fine, 1991). Fixed brains were blocked and placed in 40% (w/v) sucrose in 0.1M PB for 2 days for cryoprotection of freezing microtome sectioned tissue. Those sections from animals in group B which were processed for fluorescence microscopy were handled similarly except cryoprotection was not necessary for vibratome sectioning. Sections were cut to a thickness of 40μm. Every fifth section was collected throughout the full extent of the substantia nigra as well as through the rostral, middle and caudal striatum. Mesencephalic and

striatal sections were incubated in a mouse monoclonal anti-tyrosine hydroxylase antiserum (Incstar) (1:1000) in 0.1 M phosphate buffered saline (PBS) for 72 hours at 4°C. This incubation was followed by repeated rinses with PBS and a 2 hour incubation with biotinylated secondary antiserum, goat anti-rabbit IgG (1:250). Sections were then processed for 1 hour with 0.6% (v/v) avidin-biotin-peroxidase complex (Vector) which was reacted with 0.05% (w/v) diaminobenzidine (DAB) and 0.03% (v/v) hydrogen peroxide to produce a brown reaction product.

Sections from animals in group B were processed for fluorescence imaging to permit simultaneous imaging of TH-IR and beads, and were treated identically with the following exceptions. These brains were not cryoprotected and were cut using a vibrotome to avoid freezing of the fluorescent beads and loss of fluorescence. Primary antiserum was rabbit anti-TH (1:2000, Eugene Tech) and secondary antiserum was fluorescein labelled goat anti-rabbit IgG (1:200). This treatment was necessary in order to maintain the fluorescence of the latex microspheres.

To determine the correlation between the size of the striatal lesion with the number of cells in the SNc, the area of the striatum in one rostral and one cuadal section was measured using a BioRad SOM image analysis system.

Camera lucida drawings were prepared from every fifth section through the rostro-caudal extent of the SN at a total magnification of 40x for all DAB stained sections. The third cranial nerve was used as the medial border of the substantia nigra pars compacta (SNc), delineating it from the ventral tegmental area (VTA).

TH-IR cell counts were performed on the lesioned and control sides, at a total magnification of 160x. Sections through the ventral mesencephalon of Group B brains were observed by epifluorescence microscopy, using rhodamine and fluorescein filter sets or in BioRad MRC-500 laser scanning confocal microscope; cells with TH-IR only, beads only and beads and TH-IR were counted. All cell counts were corrected using the method of Abercrombie (Konigsmark, 1970).

Areas of cell bodies from the lateral aspect and core region of the SN of groups A ($n=9$) and D ($n=4$) were measured using the BioRad SOM video image analysis software. One field from each of these regions was randomly selected using a 16x objective. Lateral and core areas of the SNc were defined on the untreated side by extending a line from the medial edge of the nucleus to the most lateral cell. This line was traced to the opposite side from the corresponding medial border. The border between lateral and core was set two thirds of the distance from the medial extent of the line. Cells medial to that point were considered core cells. All cells in one field (core and lateral SNc) were analyzed in each section. Somal areas were measured in μm^2 by tracing the boundary of the cell body which was defined by the exclusion of the neurites beyond the point of inflection of the somal membrane.

Volumes of the SNr were determined using the same image analysis system. The medial aspect of the SNr was determined by a line drawn from the medial aspect of the SNc perpendicular to the brain surface and the lateral aspect by a similar perpendicular line from the most lateral aspect of the SNc and

extending to the brain surface. The inclusion of the cerebral peduncle in this measurement would underestimate the changes in volume of the SNr and therefore decrease the significance.

3.3 Results

Six weeks after ibotenic acid injection into the striatum, massive degeneration of the strucure was observed (Fig. 6). Linear regression analysis yielded a highly significant correlation between SNc cell number and the size of the striatal lesion (correlation coefficient = 0.7546; P value = 0.0002)(Fig. 7). This striatal degeneration led to retrograde and anterograde changes in the ipsilateral SNc and the SNr respectively in all of the lesioned-only animals. Substantial TH-IR cell loss was observed in the SNc mainly in the lateral aspect of the nucleus and a decrease in overall volume of the SNr was evident (Fig. 8). TH-IR cells of the SNc were counted in every fifth section throughout the rostro-caudal extent of the substantia nigra for groups A (n=9), C (n=6) and D (n=6) yielding a corrected count 1764 $\pm$ 101 TH-IR cells per SNc in control animals and on control sides of lesioned animals . Counting of these cells in group A revealed a significant decrease in number in the SNc ipsilateral to the lesioned striatum, 1324 $\pm$ 75, when compared with the SNc of intact controls (Fig. 9). TH-IR cell counts in the SNc contralateral to the lesion did not differ from control. When embryonic rat striatalprimordia were grafted into the striatum 2 weeks after lesioning and allowed to grow for 6 weeks. the number of TH-IR cells in the

Figure 6. Low power micrograph of a section through the unilaterally lesioned striatum of a Group A animal, stained for TH-IR. Note the massive decrease in area of the lesioned vs. the intact striatum (st). Scale bar represents 850μm. (cc, corpus callosum; L, ibotenic acid lesion)


CC
L

Figure 7. Graph showing the correlation between the number of cells in the SNc and the area of the lesioned striatum. The correlation coefficient is 0.7546 and the P value is 0.0002 yielding a highly significant correlation. The 95% confidence limits for the regression line (solid) are shown as dotted curves.

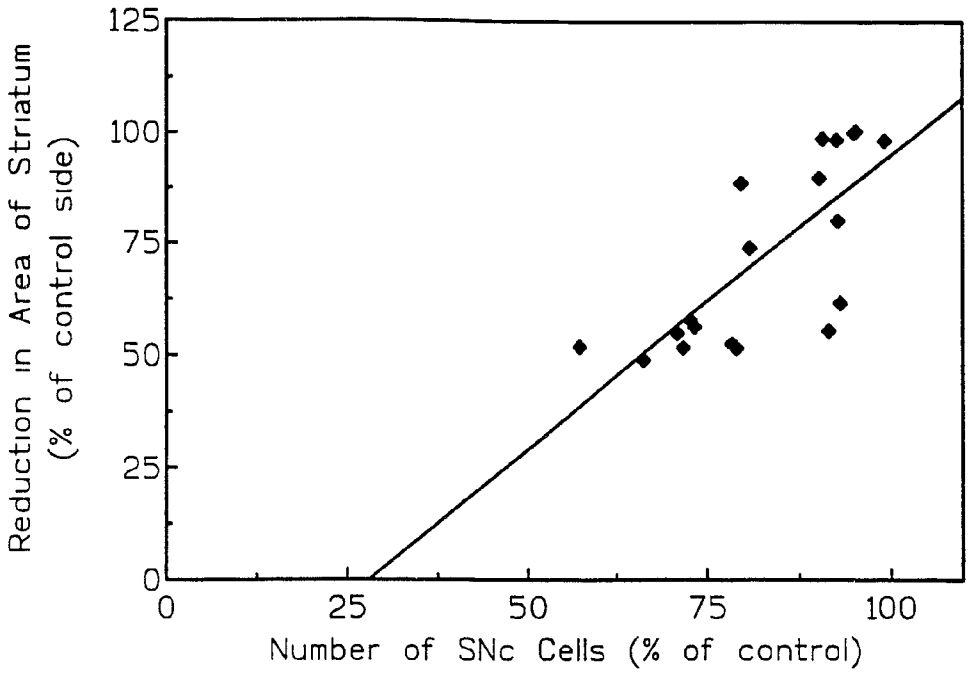

Reduction in Area of Striatum
(% of control side)
125
100
75
50
25
0
0
25
50
75
100
Number of SNc Cells (% of control)

Figure 7

Figure 8. Camera lucida drawing through the rostro-caudal extent of the substantia nigra. Each dot represents 4 cells. Dashed line demarcates the inclusive area of the SNr and cerebral peduncle. Numbers at right indicate levels of these coronal sections, in mm, posterior to bregma, as determined by comparison with the atlas of Paxinos and Watson (1986). Lesion refers to target lesion in the striatum. (cp-cerebral peduncle; nIII, third cranial nerve; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; VTA, ventral tegmental area; 3V, third ventricle)

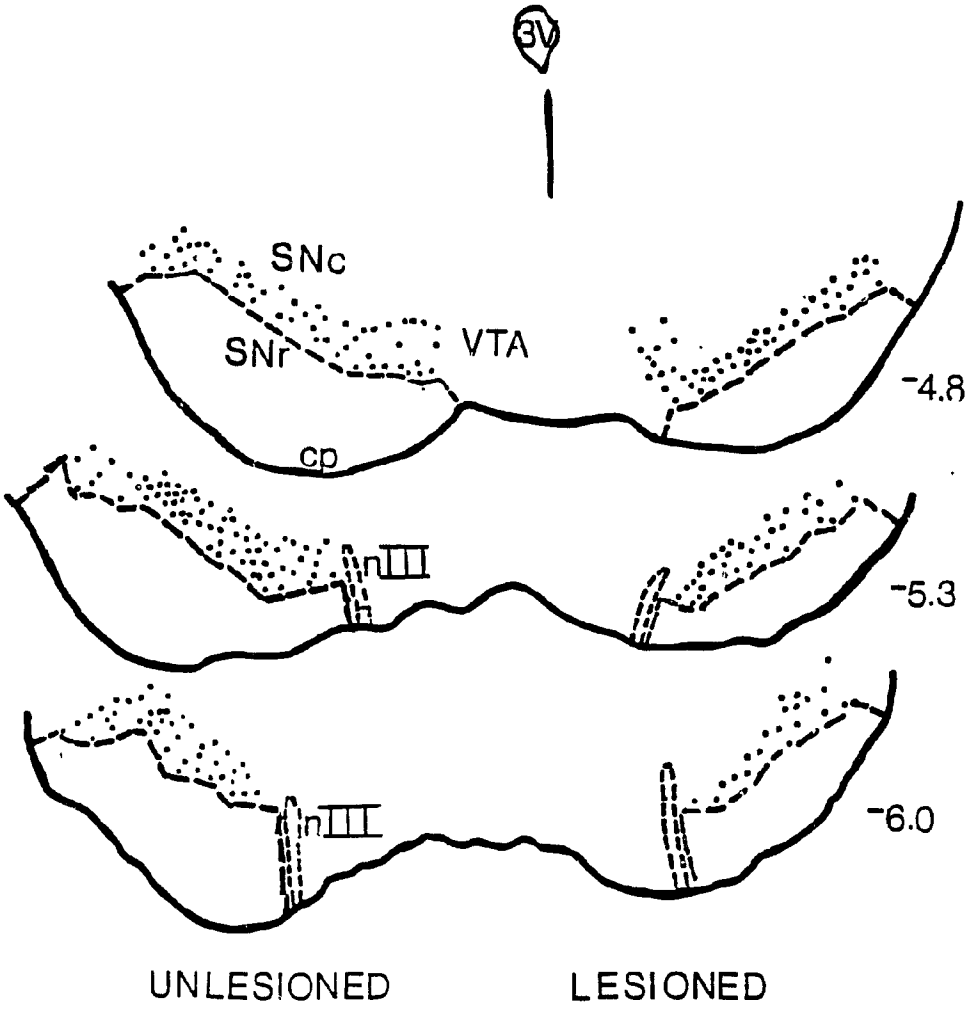


Figure 8

ipsilateral SNc increased to an intermediate level that was not significantly different from either the lesioned or control animals, 1476 ± 228. However, the number of TH-IR cells from control and lesioned animals remained significantly different ($p<0.05$) (Fig. 9). Although grafting of fetal striatal primordia into the lesioned striatum appeared to decrease the striatal lesion-associated decrease in SNc TH-IR cells, it had no effect on the lesion-associated shrinkage of the SNr (Fig. 10).

Measurements of cell soma areas from the lateral and core aspects of the SNc in lesioned and naive control animals revealed differences in cells size depending on the area of the SN that the cells are located (Table 1). Cells from the core region were significantly smaller than those residing in the lateral aspect ($p<0.05$) in all animals examined. However, there was no significant difference in cell size when comparing TH-IR cells from the SN ipsilateral to the lesioned striatum or from those of control animals.

Fluorescent retrograde tracer studies were undertaken in order to determine whether the striatal lesion-associated decrease in SNc TH-IR cell numbers reflected death of these cells or reduced TH expression in surviving cells. Prior to lesioning, injection of fluorescent latex beads into the striatum resulted in labelling of neurons in the SNc. After fluorescent immunocytochemical staining for TH 1 week post-lesioning, approximately one third of the cells throughout the entire extent of the SNc appeared double labelled with fluorescent latex beads (Fig. 11). In the control SNc ($n=8$), contralateral to the lesioned striatum, 0.43%

Figure 9. Graph showing the decrease in TH-IR cell number in the substantia nigra six weeks following an unilateral striatal lesion ($p<0.05$). Grafting of fetal striatal primordia into the striatum 2 weeks post-lesion increased the number of cells which did not differ significantly from control or lesion.

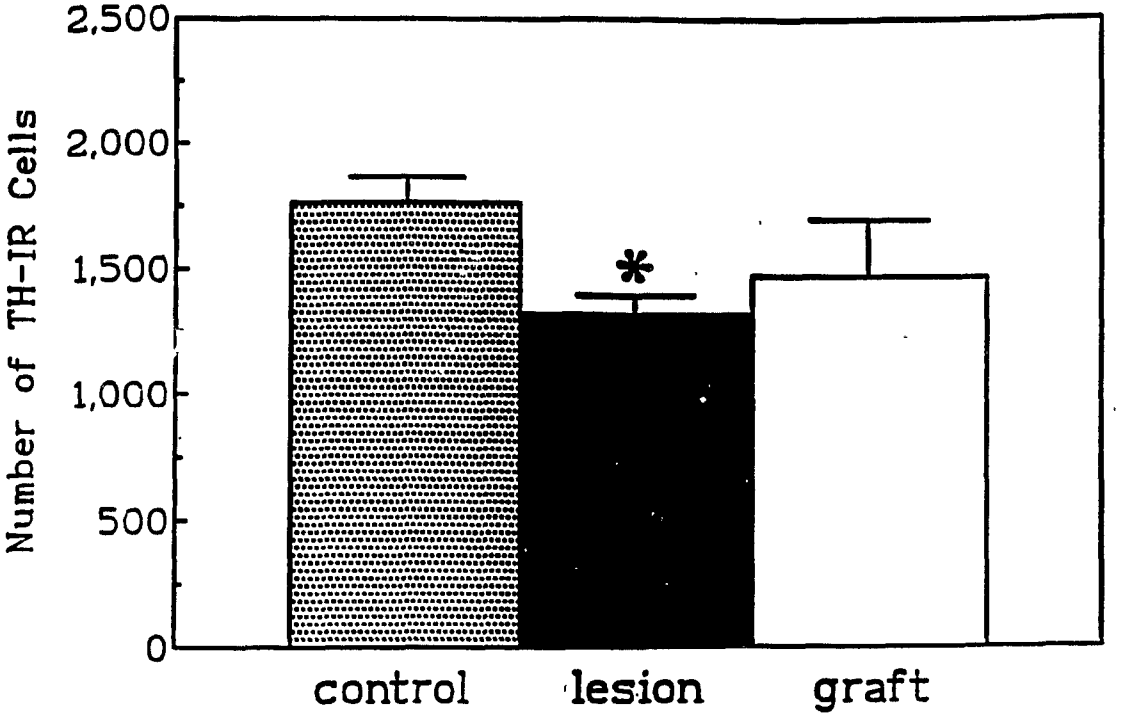

2,500
2,000
1,500
1,000
500
0
Number of TH-IR Cells
*
control
lesion
graft

Figure 9

Figure 10. Graph showing a significant decrease in the area measurements of the target lesioned SNr when compared with control v ,lues. Grafting of fetal striatal primordia to the lesioned striatum did not increase the SNr area. *, different from control, $p < 0.001$.

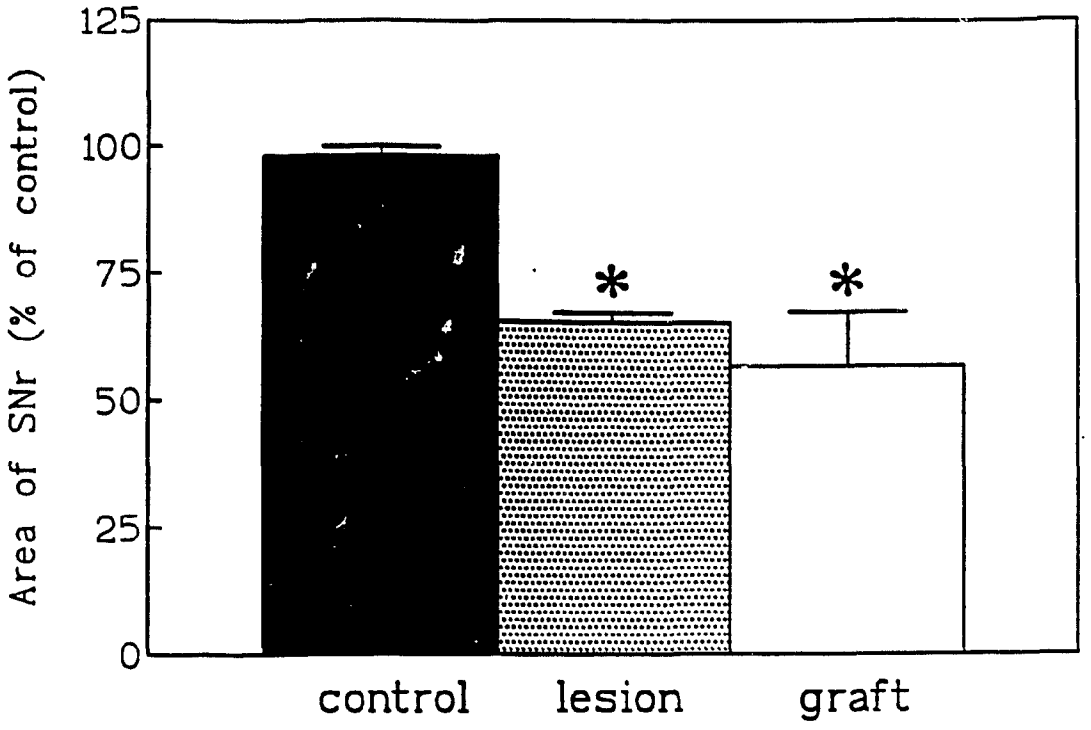
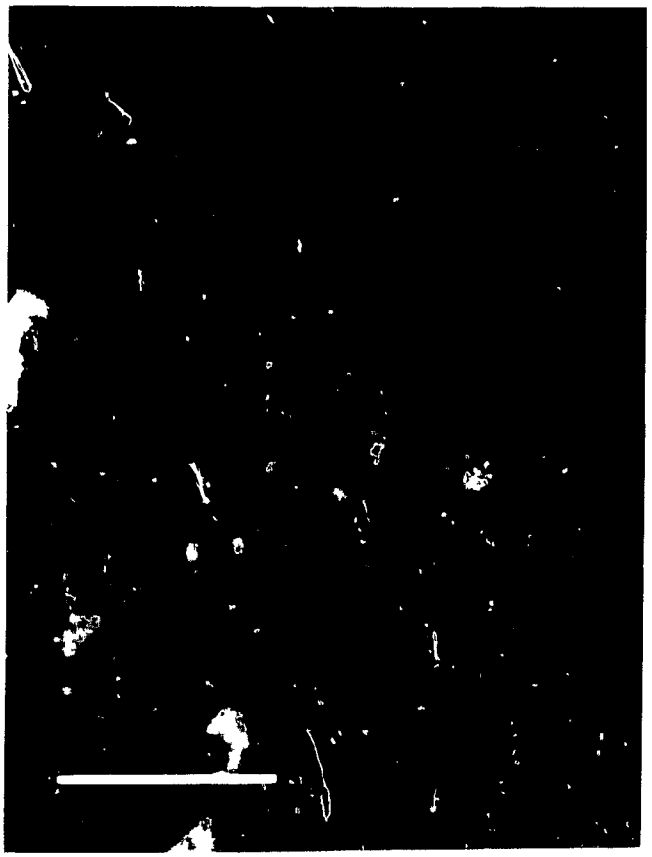


Figure 10

Figure 11. Simultaneous dual-wavelength confocal micrograph of fluorescein-labelled TH-IR cells retrogradely labelled with red fluorescent latex beads. The beads were injected into the ipsilateral striatum 3 days before ibotenic injection to the striatum and the animal was killed 6 weeks later. Scale bar is 50 μm.



11

± 0.02% (S.E.M.) of the cells were labelled retrogradely with the fluorescent beads but did not stain for TH-IR (Fig. 11). On the experimental side, however, 3.19% ± 0.16% (S.E.M.) of the cells did not stain for TH yet were labelled with the retrogradely transported beads, a difference that was highly significant ($p<0.0001$). This indicates that the labelled cells had not died but simply lost their ability to express the neurochemical marker, making them potentiallyrecoverable within some undetermined timeframe as has been shown by Blanchard *et al.* (1994), who demonstrate that dopaminergic cells lose their ability to express TH several days or weeks before death in an *in vitro* environment.

TABLE 1

Cell soma area measurements of left and right lateral and core ventral mesencephalic dopaminergic neurons from ibotenic acid striatal lesioned and control rats. Ipsilateral refers to TH-IR on the side of the striatal lesion. *'Lesioned' counts represent a mean of 325 cells in 8 animals. 'Control' counts represent a mean of 96 cells from 3 animals.*
Full details of measurements are given in the text.

	left lateral ($\mu m^2 \pm$ S.E.M.)	left core ($\mu m^2 \pm$ S.E.M.)	right core ($\mu m^2 \pm$ S.E.M.)	right lateral ($\mu m^2 \pm$ S.E.M.)
Lesioned (n=9)	241.6 ± 4.93 (ipsilateral)	206.0 ± 4.29 (ipsilateral)	208.0 ± 4.41 (contralateral)	247.1 ± 5.58 (contralateral)
Control (n=3)	222.3 ± 8.00	193.9 ± 7.04	212.8 ± 8.64	230.5 ± 7.43

3.4 Discussion

These results indicate that ibotenic acid lesioning of the striatum leads to a retrograde degeneration of the ipsilateral substantia nigra, with a 25% loss of TH-IR cells in the SNc (Fig. 9), and also to anterograde degeneration, a 35% decrease in area of the SNr (Fig. 10), 6 weeks after lesioning the striatum. Our observation of cell loss in the SNc following ipsilateral excitotoxic lesion of the striatum is in contrast to the reports of Lundberg *et al.* (1994) but is consistent with findings by others (Pasinetti *et al.*, 1991; Krammer, 1980). It should be noted that we obtained total SN TH-IR cell numbers substantially lower than other published observations of 3,500-4,000 cells per intact SNc (Anden *et al.*, 1966; Bowenkamp *et al.*, 1995), presumably a consequence of the arbitrary SN boundaries we imposed that likely excluded cells in adjacent zones counted by others.

The results of latex bead injection studies indicate that a small population of SNc cells that project to the striatum do not normally express TH. This population of cells has been estimated to comprise 1-10% of the SNc neurons projecting to the striatum (Gerfen *et al.*, 1985). Here, we observed a far smaller percentage, 0.43%, of such SNc cells in the SN contralateral to the lesioned striatum, but found that this fraction increased ipsilateral to the striatal lesion. A possible explanation for this low number is that the lesioned striatum is exerting not only an effect on the ipsilateral SN but also a contralateral effect on the unlesioned side since no counts were made in naive animals. Also, the TH antiserum may not have penetrated the 40μm section in order to bind adequately

to the enzymes of all TH containing cells rendering an underestimate of the actual number of TH-negative cells. Identical treatment of all sections however nullifies this effect. Bead labelled/TH-negative cells may represent dopaminergic cells no longer expressing TH or non-dopaminergic projecting cells that may be particularly susceptible to target lesions and, therefore, preferentially compromised. This increase in bead positive-TH negative cells following striatal lesioning, however, most likely represents formerly dopaminergic cells no longer capable of expressing TH.

Such injured but still viable cells might be restorable to their former state by various interventions. Attempts have been made to recover cells lost in the SNr due to ibotenic acid striatal lesions (Schallert *et al.*, 1990; Janson *et al*, 1988). Here, when fetal striatal primordia were grafted into the striatum within days of lesioning, lesion associated cell death was reduced after 6 weeks of growth, suggesting that SNc cell death after striatal excitotoxic lesions may result from subsequent loss of retrogradely transportable striatal-derived trophic factors such as have been previously described (Prochiantz *et al.*, 1979; Tomozawa and Appel, 1986; Dal Toso *et al.*, 1988) or a specific, yet unidentified environmental signal (Janson *et al.*, 1988; Friedman *et al*, 1988; Saji and Reis, 1987). After grafting fetal stiatal primordia into the lesioned striatum, it is conceivable that the spared terminals of SN projecting neurons are able to retrogradely transport sufficient levels of trophic factors to save some TH-IR cells that would have otherwise been lost.

In contrast, the loss in volume of the SNr was not affected by these grafts. Since a major neuronal projection to the SNr are GABAergic striatal cells, the killing of these cells by ibotenic acid lesions, could result in extensive cell death or atrophy in the SNr. Since fibre outgrowth from grafted fetal cells is limited to only a couple of millimetres, the possibility of reestablishing connections with the SNr is remote. Since this loss in volume of the SNr indicates loss of cells and probably loss of fibres from the caudal projecting GABAergic striatal cells, it is unlikely that replacement of fetal striatal cells into the striatum would affect the SNr. However grafting of fetal striatal primordia into the SNr may enhance cell survival if this loss is due to some cell death and not only fibre loss, and if functional connections are formed.

Morphometric analysis of the cell soma size indicated that cells in the lateral and core areas of the SNc are significantly different (Table 1). Cells from similar locales however did not differ significantly in size between the control and lesioned animals. The area of the cell soma actually tended to *increase* in size although not significantly, following ibotenic acid striatal lesions (Table 1). These results do not support the observations of Lundberg *et al.* (1994) who found that after lesioning, TH-IR cell bodies in the SNc were reduced in size. In our study, a representative population was randomly selected from each division of the nucleus and the area of all cells in the field was measured. Lundberg *et al.* (1994) reported the retrograde labelling of cells in the SNc using a small localized tracer injection into the CPu followed by an ibotenic acid lesion of the same

nucleus; the somal areas of all labelled cells in the SNc were measured and found to decrease after lesioning. Such labelling presumably included cells that do not express TH-IR, and that would have been excluded by our methods. Indeed, SNc cells that decreased in size and also ceased TH expression would be omitted from our analysis, whereas cells that maintained TH expression may actually hypertrophy after the lesion, perhaps as part of a sprouting response to dennervated targets deafferented by the degenerating (dead or non-TH-expressing) cells.

Thus, the TH-IR cells of the SNc are sensitive to the health of their striatal target neurons. Within weeks of lesioning their target, TH-IR cells in the SNc decrease in number. Some of these cells may be rescued if grafts of fetal striatal primordia are subsequently transplanted into the recently lesioned striatum. These findings are consistent with the possibility that cells of the SNc die in response to the loss of trophic support from the striatum, not simply due to axonal injury during the lesioning. Identification of the trophic factor(s) may have consequences for the treatment of a number of basal ganglia disorders.

4.0 Response of Human Fetal Ventral Mesencephalic Dopaminergic Neurons to Identified Trophic Factors *in Vitro*

4.1 Introduction

Dopaminergic ventral mesencephalic neurons from the substantia nigra (SN) decrease in number in Parkinson's disease (PD). The factor(s) responsible for this decline are still unknown: manganese (Cook *et al.*, 1974), carbon monoxide (Ringel and Klawans, 1972), cyanide (Qitti *et al.*, 1985) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Calne *et al.*, 1985) may cause parkinsonian symptom, but none of these toxins is likely to be responsible for classical idiopathic PD, a slowly progressive degenerative disease. It is of interest in this context to identify factors that influence dopaminergic SN cell survival over extended periods of time Peptide growth factors which are normally present within the basal ganglia and are responsible for the growth and survival of neurons during development and maintaining adult neurons throughout life are important candidates. Identification of such factors that enhance the survival of human dopaminergic neurons *in vitro* may have therapeutic value possibly slowing the progression of the disease.

Nerve growth factor has been shown to support the *in vitro* survival and development of cholinergic cells from the basal forebrain (Honegger and Lenoir, 1982, Gahwiler *et al.*, 1987; Hatanaka *et al* , 1988; Hartikka and Hefti, 1988;

Lapchak and Hefti, 1992) but has no effect on dopaminergic neurons (Dreyfus *et al.*, 1980). Other members of the neurotrophin family of growth factors, however, can support the survival of dopaminergic mesencephalic neurons. Brain-derived neurotrophic factor (BDNF) can enhance survival of rodent ventral mesencephalic dopaminergic neurons *in vivo* and *in vitro* (Hymen *et al*, 1991; Lindsay *et al.*, 1993; Beck *et al.*, 1993) and its retrograde transport and colocalization within TH-IR cells in the substantia nigra have been demonstrated (Mufson *et al.*, 1994). After lesioning, decreased levels of BDNF have been reported (Vereno, 1994), suggesting its expression by substantia nigra dopaminergic neurons. Cell survival was supported, neurite outgrowth enhanced (Studer *et al.*, 1995) and electrical activity (Shen *et al.*, 1994) increased along with dopamine uptake. Its effect on human fetal dopaminergic cells, however, has been reported by one group who described a 2.2-fold increase in TH-IR cell survival and a doubling of dopamine production (Zhou *et al.*, 1994).

The heparin binding factor, basic fibroblast growth factor (bFGF), has been shown to be expressed in the pigmented cells of the substantia nigra at a level which is unaffected by age in the normal human brain. In PD, however, approximately 13% of these previously bFGF positive pigmented cells express the factor (Tooyama *et al.*, 1994). Bean *et al* (1991) have described bFGF mRNA in the SN of rat, monkey and human. In the rat, TH-IR cells in the substantia nigra also express cytoplasmic bFGF-IR (Cintra *et al.*, 1991) and anterograde transport to the striatum has been described (McGeer *et al.*, 1992). bFGF has been shown to

exert trophic effects on survival of dissociated rat ventral mesencephalon neurons in culture by some investigators (Casper *et al.*, 1994) but not others (Hartikka *et al.*, 1992) and weak trophic effects, increasing TH-IR cell survival by 65% with 50 ng/ml bFGF, apparently mediated through glial cells, have been reported (Silani *et al.*, 1994). Specific heparan sulfate proteoglycan epitopes have been shown to be present in high concentration in the substantia nigra, possibly indicating a bFGF storage site within the extracellular matrix (Fuxe *et al.*, 1994). Heparin binding with growth factors other than FGFs has not been described.

Recently, disruption of the IGF-I gene has been shown to reduce the brain size in mice although the number of mesencephalic dopaminergic neurons remained unaltered (Beck *et al.*, 1995); however, addition of IGF-I to culture medium has been shown to support the survival of cholinergic and dopaminergic neurons *in vitro* (Knusel and Hefti, 1991). IGF-II, when incubated with rodent ventral mesencephalic dopaminergic neurons, was found to promote growth of cell bodies and neurites but to have no effect on cell number (Liu and Lauder, 1992).

Here I show that the *in vitro* increase in number of ventral mesencephalic dopaminergic neurons from 6 to 10 week gestational age human fetuses is greatly enhanced by heparin supplemented bFGF and only moderately by bFGF or BDNF, while IGF-I and IGF-II do not affect survival of these dopaminergic neurons in vitro under the conditions described here.

4.2 Materials and Methods

Human fetal ventral mesencephalon was dissected from aspirated fetal tissue of 6-10 week post-conception age obtained from elective abortions performed at the Victoria General Hospital, Halifax, NS, with informed maternal consent and institutional review board approval. Age of the fetuses was determined by comparison of foot and hand development with standard references (England, 1983). The ventral aspect of the mesencephalic flexure was isolated and the meninges were removed. For each experiment, ventral mesencephalon from up to 3 fetuses was pooled. In cases where sufficient material was not available on the same day, tissue was stored in "hibernation medium" for up to 5 days at 4°C; hibernation medium, consisting of an aqueous solution containing 0.22% (w/v) potassium chloride, 0.09% (w/v) glucose, 0.005% (w/v) magnesium chloride, 0.013% (w/v) monobasic sodium phosphate, 0.071% (w/v) dibasic sodium phosphate, 0.6% (v/v) lactic acid, 0.18% (w/v) potassium hydroxide and 3.1% (w/v) Sorbitol was prepared and utilized as previously described (Sauer and Brundin, 1991).

Cells from the human ventral mesencephalon were dissociated and cultured as described in section 2.2. Briefly, the tissue was cut into pieces measuring approximately $1mm^3$, pooled and incubated for 20 minutes at 37°C in 0.05% (w/v) trypsin (type XIII)(Sigma) in CMF-HBSS solution. The tissue was then rinsed 3 times in 0.01% (w/v) deoxyribonuclease I (Sigma) in complete HBSS and

then resuspended in a minimal volume of CMF-HBSS. The digested tissue fragments were then dispersed by repeated gentle pipetting using five fire-polished pipettes with bore size of decreasing diameter. When the cells were completely dispersed to a single cell suspension, they were counted using a hemocytometer and plated in chemically defined medium at a density of 2 x 10^5 cells/cm^2 on poly-L-lysine (Sigma) coated Nunc 24 well culture dishes.

The chemically defined medium consisted of sodium bicarbonate buffered Dulbecco's modified Eagle medium and Ham's F-12 (3:1) with the following additives: insulin 25μg/ml, human transferrin 100μg/ml, progesterone 20nM, putrescine 60nM and sodium selenite 30nM, all purchased from Sigma. Each experiment consisted of a triplicate plating of cells at the growth factor concentrations of 100, 50, 20, 10, 5, 1, 0.1 and 0ng/ml and were converted to molarity for analysis. Where heparin was added, its concentration was 10μM in all cases. The growth factors tested were BDNF (Genentech, USA), bFGF (Upstate Biochemicals, Lake Placid, NY), IGF-I (Upstate Biochemicals, Lake Placid, NY) and IGF-II (Upstate Biochemicals, Lake Placid, NY). BDNF was reconstituted in ethanol, bFGF in Tris buffered saline pH 7.4, IGF-I in 0.1M acetic acid and IGF-II in DMEM. Equal volumes of vehicle were maintained throughout all of the concentrations. Al[1] cultures were grown for 10 days at 37°C in a 5% CO_2 incubator and were fed every 3 days by gentle replacement of half of the medium with fresh medium and growth factors.

At the end of the culture period, cells were fixed with 4%

paraformaldehyde in 0.1M phosphate buffer pH 7.4 for 20 minutes and stained immunohistochemically for tyrosine hydroxylase. After fixing, the cells were washed 3 times with 0.01M phosphate buffered saline (PBS) containing 0.3% (v/v) Triton X-100 (Sigma) with the second wash containing 0.3% (v/v) hydrogen peroxide. Non-specific staining was then blocked by incubating cells for 1 hour at room temperature with PBS containing 10% normal goat serum (NGS). This was followed by an overnight 4°C incubation with primary rabbit anti-tyrosine hydroxylase (1:2000)(Eugene Tech) in 1% NGS and 0.1% Triton X-100 (v/v) in PBS. The cultures were then rinsed 3 times and incubated for 1 hour at room temperature with biotinylated goat anti-rabbit IgG (1:250) (Penninsula Labs). After 3 rinses with PBS, an avidin-biotin complex (Vectastain) was incubated with the cultures for 1 hour at room temperature: the cultures were then rinsed 3 times with PBS and incubated with 0.05% (w/v) DAB in PBS for 5 minutes, which hydrogen peroxide was added (final concentration, 0.015% v/v). TH-positive cells were stained dark brown; all stained cells on the culture dish were counted using an inverted microscope with a 16x objective.

Each candidate trophic factor was tested 4 times in triplicate, with a given ventral mesencephalic preparation used to generate samples at each test concentration. The number of TH-positive cells in each case is expressed as percent of mean cell number in the control since the number of TH-positive cells recovered in each fetal sample was variable by the nature of the tissue and its recovery. Means were compared using analysis of variance (ANOVA).

4.3 Results

The ventral mesencephalon of 35 human embryos between the ages of 6 and 10 post conception weeks were recovered. The tissue was enzymatically digested to form a single cell suspension which grew well for at least 10 days in chemically defined cell culture medium on poly-L-lysine-coated tissue culture dishes. TH-immunoreactive neurons were easily identified using a polyclonal antibody to tyrosine hydroxylase (Fig. 12). Cell culture medium supplemented with 14.3 nM bFGF yielded significantly more TH-immunoreactive cells (by 89.5 $\pm$ 12.0%) than did control medium ($p<0.001$; Fig. 13). With the addition of heparin (10μM) to the bFGF supplemented medium, a much greater effect was observed (Fig. 13), to an increase of 340.3 $\pm$ 36.9% in TH-immunoreactive cell yield with the addition of 14.3 nM. This increase began to rise sharply between 1.15 and 5.73 nM and began to plateau at the highest concentration tested of 28.6 nM. The difference from control was significant ($p<0.01$) of concentrations $\geq$1.43 nM.

BDNF had only a modest effect on the survival of TH-immunoreactive cells (Fig. 13), yielding cell numbers 102.9% $\pm$ 14.5 above control at concentrations $\geq$1.43 nM, with no effect seen below 1.43 nM.

The addition of IGF-I or IGF-II to the culture medium did not alter the survival of human TH-immunoreactive cells in this study (Fig. 13).

Figure 12. Human ventral mesencephalic TH-IR cells after 10 days in vitro in chemically-defined medium with no trophic factors added. Scale bar is 75μm.

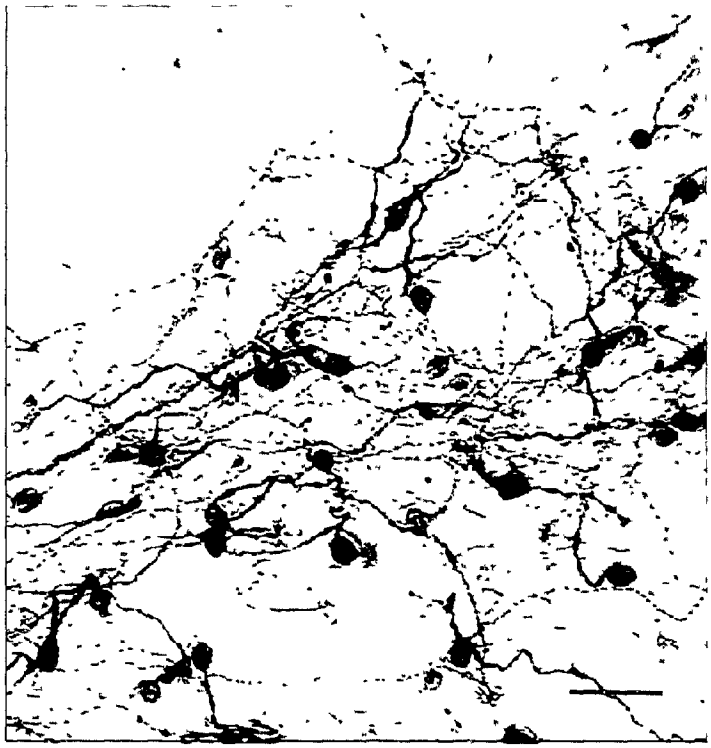


Figure 12

Figure 13. The effect of 10 day exposure to varying concentrations of candidate trophic factors upon the numbers of surviving human fetal ventral mesencephalic TH-IR cells *in vitro*. Points represent the number of TH-IR cells as a percent of control. Vertical lines represent S.E.M. Dashed line represents no added trophic factor for each experiment.

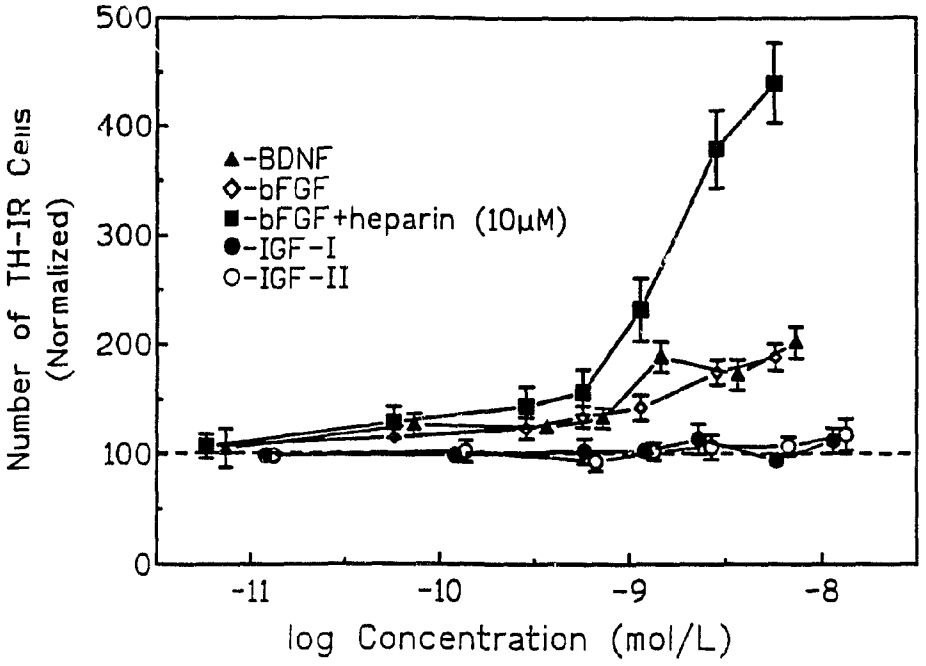

500
400
300
200
100
0
Number of TH-IR Cells
(Normalized)
▲-BDNF
◇-bFGF
■-bFGF+heparin (10μM)
●-IGF-I
○-IGF-II
-11
-10
-9
-8
log Concentration (mol/L)

Figure 13

4.4 Discussion

In this study, I have shown that human ventral mesencephalic dopaminergic neurons are responsive to specific peptide growth factors by increasing the survival of TH-IR cells *in vitro*. The effects of bFGF and BDNF alone were moderate but in combination with heparin, 28.6 nM bFGF exposure resulted in a 4.5 fold increase in dopamine cell survival; however heparin alone did not enhance the survival of human fetal dopaminergic neurons. The IGFs did not exert any affect on cell survival in this system. Because heparin binding has not been demonstrated with non-FGF growth factors, experiments with heparin and BDNF or the IGFs were not attempted.

These results corroborate observations by Spenger *et al.* (1995) who reported that, when grown in roller culture, TH density of human dopaminergic neurons is enhanced 2 fold with exposure to 2.86 nM BDNF. The differing culture techniques between Spenger *et al.* (1995) may be the basis of the slight difference in survival of human dopaminergic neurons since substrate penetration is typically enhanced and attachment inhibited in roller culture. For these reasons, fetal dopaminergic cells in this case may exist in a slightly altered state of differentiation and therefore may respond differently to BDNF. Silani *et al.* (1994) have described enhanced survival of human dopamine neurons by 65% *in vitro* with bFGF but no co-incubation with heparin was performed.

The greatest concentration of BDNF in the central nervous system has been

found in the adult and developing hippocampus and cortex (Maisonpierre *et al.*, 1990; Friedman *et al.*, 1991b). Low levels of BDNF mRNA have also been found in the developing and adult ventral mesencephalon (Maisonpierre *et al.*, 1990b), but not in the striatum. Trophic effects of BDNF on ventral mesencephalic dopaminergic neurons *in vivo* and *in vitro* have been extensively described (Hyman *et al*, 1991; Knusel *et al*, 1991), and the colocalization of trkB tyrosine kinase receptor for BDNF with tyrosine hydroxylase has been described in the developing, but not the adult, rat ventral mesencephalon (Hynes *et al.*, 1994). At the present time however, it is not known to what extent BDNF or its receptor function in the CNS (refer to section 1.2). Klein *et al.* (1993) have genetically deleted the trkB gene and therefore the BDNF receptor; although the mice did not survive after birth, post mortem examination of the brains showed no abnormality in the substantia nigra. Thus the function of this factor is not well understood and although an increase in survival of dopaminergic neurons with BDNF was noted here, this may not be a normal influence *in vivo*.

bFGF, a trophic factor shown to be produced by dopaminergic neurons in the ventral mesencephalon (Bean *et al.*, 1991; Cintra *et al.*, 1991), has been shown to exert strong trophic effects on cholinergic neurons of the rat basal forebrain that do not seem to be mediated by any secondary cell type that may be present among cholinergic neurons (Ferrari *et al.*, 1989; Knusel *et al.*, 1990). In the ventral mesencephalon however, trophic effects of bFGF on dopaminergic cells seem to be mediated through mesencephalic astrocytes. Addition of bFGF to

astrocyte culture media has led to astrocytic synthesis and release of NGF, (Fukumoto *et al.*, 1991) which does not affect dopaminergic neurons, therefore bFGF may be inducing cells in the vicinity to produce and release factors such as GDNF or BDNF that may exert a cell survival effect back on the original cell.

bFGF has been shown to bind with high affinity to a variety of FGF receptors (Partanen *et al.*, 1992) and also with low affinity to transmembrane proteoglycan containing heparin sulfate (Kiefer *et al.*, 1990). This low affinity binding or simple addition of exogenous heparin is crucial for subsequent binding to the high affinity receptor. Expression of a high affinity FGF receptor has been shown in the substantia nigra, but the exact localization is not known (Wanaka *et al.*, 1990). Further investigation is required to determine the cellular location of the receptors during development and in the adult, in order to ascertain the locus of action for bFGF. Thus, to determine whether bFGF is acting directly on the dopaminergic neurons or indirectly via neighbouring glial cells, it could be applied to dopaminergic cells grown in a glia-free environment. With the use of serum-free medium in this study, the concentration of glia was reduced but treatment with a mitotic inhibitor is necessary to ensure an astrocyte free environment.

IGF-I and IGF-II have been shown to enhance survival and stimulate neurite outgrowth of dopaminergic cells from the ventral mesencephalon in rat (Knusel *et al.*, 1990), but no binding sites have been described in the adult (Marks *et al.*, 1991). Adem *et al.* (1989) however have shown low level binding in the

human fetal striatum and substantia nigra. In this study, I observed no significant enhancement of human dopaminergic neuron survival by these insulin-like growth factors.

The increase in number of dopaminergic neurons does not necessarily indicate an increase in cell survival but rather it could indicate an effect on cell proliferation instead. In this study, this possibility was not addressed. To distinguish between increased cell survival and induced cell proliferation, one could expose cells to BrdU during administration of the growth factors to label proliferating cells.

Other factors that have recently been shown to enhance survival or outgrowth of dopaminergic neurons, such as glial cell-line derived neurotrophic factor (GDNF) (Lan *et al.*, 1993), or possibly others that have not yet been identified, may exert profound effects on this system. Such trophic factors may account for the dramatic trophic influence of the striatum on ventral mesencephalic dopaminergic neurons *in vivo* as shown in section 3. These growth factors may be of potential use to enhance dopaminergic cell survival and cell transmission in Parkinson's disease. They could also enhance survival or outgrowth of cellular grafts of human fetal ventral mesencephalic dopaminergic neurons into the striatum of Parkinsonian patients if infused or added to the injected cell mixture, thereby possibly increasing the grafts' effectiveness.

5.0 Effects of L-DOPA on the Survival of Rat and Human Dopaminergic Neuron Survival

5.1 Introduction

Parkinson's disease (PD), a neurodegenerative disorder characterised by progressive movement impairment, results from degeneration of the dopaminergic neurons of the substantia nigra pars compacta (SNc) (Duvoisin, 1987) and the consequent dopaminergic depletion of the neostriatum. Drug therapy for PD commonly involves administration of L-dihydroxyphenylalanine (L-DOPA), the metabolic precursor of dopamine, ordinarily supplemented with the peripheral dopa decarboxylase inhibitor, carbidopa (Pinder *et al.*, 1976), to augment the depleted dopamine stores.

Recently, intrastriatal transplantation of fetal dopaminergic neural tissue has been under experimental investigation as a therapeutic possibility. Such grafts have been shown to decrease the severity of motor deficits in a 6-hydroxydopamine (6-OHDA)-lesioned rodent model of PD (Perlow *et al.*, 1979; Björklund and Stenevi, 1979). The results of these and subsequent intrastriatal fetal dopaminergic allografts in monkeys (Redmond *et al.*, 1986; Bakay *et al.*, 1987; Bankiewicz *et al.*, 1988; Fine *et al.*, 1988; Sladek *et al.*, 1988), as well as human fetal dopaminergic grafts into the dopamine-depleted striatum of immunosuppressed rodents (Stromberg *et al.*, 1986; Brundin *et al.*, 1986 and

1988), have led to the initiation of experimental trials of human fetal dopaminergic allografts for therapy of PD (Lindvall., 1988; Freed *et al.*, 1990; Lindvall *et al.*, 1994).

Despite the dramatic effect of such grafts in rodent models of PD, these first human trials have had only limited success. A conspicuous difference between the animal and human procedures is that PD patients, but not animals, have invariably been chronically medicated with L-DOPA and carbidopa even after receiving grafts. The effects of these drugs on the survival, differentiation and outgrowth of fetal dopaminergic neurons have been suggested as reasons for reduced transplant efficacy in PD patients; however, this remains controversial (Steece-Collier *et al.*, 1990; Chalmers and Fine, 1991; Yurek *et al.*, 1991; Blunt *et al.*, 1992; Mena *et al.*, 1992; Paino *et al.*, 1992; Mytilineou *et al.*, 1993; Pardo *et al.*, 1995).

We therefore examined the effects of these drugs, administered at levels comparable to human therapeutic doses (Sharpless and McCann, 1971; Ahlskog *et al.*, 1989), in 6-OHDA-lesioned hemi-Parkinsonian rats (Ungerstedt, 1971) with fetal dopaminergic grafts to the deafferented striatum. In order to characterise the precise concentration dependence of L-Dopa effects, we carried out additional *in vitro* experiments exposing rat and human fetal dopaminergic neurons to a wide range of L-DOPA dosages. These cell culture experiments also allowed more detailed morphometric analysis of drug effects. Our studies indicate that within a concentration range comparable to the therapeutic range for individuals with PD,

L-DOPA does not significantly affect the survival or outgrowth of rat ventral mesencephalic dopaminergic neurons *in vitro* or *in vivo*, nor does it alter the survival of human ventral mesencephalic dopaminergic neurons *in vitro*.

5.2 Materials and Methods

In vivo studies were carried out with male Sprague-Dawley rats (Charles River, Montreal), weighing 250g at the beginning of the experiment, maintained on a 12 hour dark-light cycle with unrestricted access to food and water. All rats were injected i.p. with 25 mg/kg desmethylimipramine (Sigma) in saline, 30 minutes prior to unilateral stereotaxic injection of 6-hydroxydopamine (6-OHDA) (Sigma) into the medial forebrain bundle. Under sodium pentobarbital anaesthesia (65 mg/kg), 5μl of 6-OHDA (2 mg/ml in 0.9%(w/v) NaCl with 0.2 mg/ml ascorbate) was injected over 5 minutes using a 30 gauge needle, which was left in position for an additional 5 minutes before removal. The stereotaxic co-ordinates for the 6-OHDA injection, according to Paxinos and Watson (1986), were: 3.6mm caudal to bregma; 1.6mm to the right of midline; 8.2mm ventral to skull surface at midline.

Rats were behaviourally screened after 4 weeks, to assess the extent of the 6-OHDA lesion. Only rats turning on average at least 5 turns/minute for 30 minutes after i.p. injection of apomorphine (0.5 mg/kg in ascorbate-saline) were used for the experiment. On the basis of apomorphine-induced turning

performance, the animals were divided into two balanced groups, the L-DOPA treated group with 12 animals and control group with 11 animals. Beginning 6 weeks after 6-OHDA injection into the substantia nigra, one group received twice-daily injections of L-DOPA (25 mg/kg) and carbidopa (2.5 mg/kg) (Merck, Sharp and Dohme, Harlow, UK), freshly dissolved in 0.9% NaCl containing 0.07% (w/v) Pluronic F-127 (Molecular Probes Inc.) by stirring briefly in a 60°C water bath and cooling to room temperature before injection. The second group received control injections at the same times, containing identical volumes of saline plus Pluronic F-127, but without L-DOPA/carbidopa. Drug treatment was initiated prior to transplantation to assure L-DOPA loading, thereby more closely modelling the human clinical situation.

Five days after L-DOPA/carbidopa injections were initiated, all rats in both groups received grafts of ventral mesencephalic tissue from 15mm crown-rump-length Sprague Dawley rat embryos into the dopamine depleted striatum. The ventral mesencephalon was dissected from 12 embryos and incubated for 20 minutes at 37°C in 0.05% (w/v) trypsin (Type XIII, Sigma) followed by four washes in ice cold 0.01% (w/v) deoxyribonuclease 1 (DNase, Sigma). Both solutions were dissolved in 0.9% saline containing 0.6% (w/v) D-glucose (glucose-saline). The embryonic ventral mesencephalic fragments were then dissociated in glucose-saline with 0.01% DNase by gentle repeated pipetting with bore sizes of decreasing diameter. Cells were suspended to a density of approximately 300,000 cells/μl. A total volume of 1μl was injected over 2 minutes into each of

two sites in the striatum ipsilateral to the prior 6-OHDA injection, using a 10μl syringe (needle O.D. 250μm, I.D. 150μm), at the following co-ordinates according to Paxinos and Watson (1986): 0.5mm rostral to bregma, 3.0mm to the right of midline and 4.5 and 6.5mm ventral to skull surface at midline; after each injection the needle remained in place for an additional 5 minutes before withdrawal. Grafting was performed on animals in the two groups in a random sequence. Drug and saline injections were continued for 8 weeks according to the same injection protocol established before transplantation. To avoid L-DOPA-induced seizures, the drug doses were reduced to 10 mg/kg L-dopa and 1 mg/kg carbidopa the day after transplantation for ten days, after which the dose was increased to 15 mg/kg L-DOPA and 1.5 mg/kg carbidopa, the highest subconvulsant dose tolerated by these animals.

Eight weeks after grafting, all animals were perfused transcardially with 200 ml of ice cold 0.1M phosphate buffer pH 7.4 (PB), followed by 500 ml of ice cold 4% paraformaldehyde in PB. Brains were removed and postfixed in 4% paraformaldehyde solution overnight at 4°C. They were then cryoprotected in 30%(w/v) sucrose in PB at 4°C. Frozen 50 μm thick sections were cut using a freezing microtome, and every fifth section through the striatum stained for tyrosine hydroxylase (TH) immunoreactivity as a marker of dopaminergic neurons. Sections were incubated for 1 hour at room temperature in 10% normal goat serum in 0.01 M PB containing 0.3% Triton X-100 then for 72 hours at 4°C in a mouse monoclonal anti-TH antibody (Incstar, 1:1000) in PB containing 1% (v/v)

normal goat serum (NGS) and 0.3% (v/v) Triton X-100. Secondary antibody incubation followed with a 2 hour incubation at room temperature in biotinylated goat anti-mouse IgG (Jackson, 1:50). Bound antibody complex was detected using avidin-biotin-peroxidase (Vector) and 0.05% (w/v) diaminobenzidine (DAB, Sigma) reacted with 0.03% (v/v) hydrogen peroxide.

To measure the survival of grafted dopamine cells, all TH-immunoreactive (TH-IR) cells in every fifth section throughout the rostral-caudal extent of the graft were counted using a final magnification of 100x, and the total number calculated using the correction of Abercrombie (Konigsmark, 1970). TH-immunoreactivity was further analyzed quantitatively using an image analysis system (SOM, BioRad). An image of the section was displayed on a video monitor; the area of the graft-derived fibre outgrowth (graft-innervated area) including the cellular graft itself was delimited using a computer mouse, and automatically computed for each section in constant arbitrary units. Areas from sequential sections were summed to yield total graft-innervated volumes, in arbitrary units. To measure the density of graft-derived dopaminergic innervation mean optical density of graft-innervated areas (including the graft) is expressed as a percentage of mean optical density of the unlesioned contralateral striatum in the same section (to correct for section-to-section variation in staining intensity); mean optical density of the entire striatum on each side was also determined, in order to minimise possible sampling bias. All assessments were performed on coded sections without knowledge of the experimental group to which the animal belonged.

Student's t-test was used to compare the means of the L-DOPA and saline treated groups.

For *in vitro* studies of L-DOPA toxicity, cells from the ventral mesencephalon of rat embryos of 15mm crown-rump-length (n = 16) were prepared as described above. Cells were plated in 30mm cell culture dishes (Falcon) which had been previously treated with poly-L-lysine (Sigma, 0.1 mg/ml in water) at a density of 10,000 cells/cm² in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum. L-DOPA, dissolved in Hanks' balanced salt solution (HBSS) containing Pluronic F-127 (0.1% w/v), was added to the culture dishes in triplicate beginning the second day of culture to yield final L-DOPA concentrations ranging from $1x10^{-9}$M to $1x10^{-5}$M and a constant final Pluronic F-127 concentration of 0.001% (w/v). Controls had equivalent addition of HBSS only, or of HBSS with 0.001% w/v Pluronic F-127, to the culture medium. The culture medium was changed every 2-3 days. After ten days *in vitro* all cultures were rinsed twice with HBSS and fixed with 4% paraformaldehyde in 0.1M PB for 30 minutes at room temperature.

Additionally, human fetal ventral mesencephalon tissues from 6-9 weeks gestational aged fetuses (n = 13), were obtained from elective abortions performed by aspiration at the Victoria General Hospital, Halifax, Nova Scotia, with informed maternal consent and institutional review board approval. All tissue was maintained in a cooled environment immediately following the removal of the tissue until enzymatic treatments. The ventral mesencephalon was identified and

dissected from the ventral mesencephalic flexure and dissociated and plated in duplicate on poly-L-lysine coated 24 well tissue culture dishes (Nunc) at concentrations of L-Dopa ranging from 10^{-11} to 10^{-4}.

All cultures were stained immunohistochemically with either rabbit antiserum against tyrosine hydroxylase (Eugene Tech)(human and rat cultures) or mouse monoclonal A2B5 antibody (Seralab)(rat cultures only). The A2B5 antibody, which recognises a tetrasialoganglioside on the plasma membrane of neurons, oligodendrocytes and type-2 astrocytes (Raff et al., 1983), served to identify non-specific, as opposed to dopaminergic toxicity in rat cultures. All cultures were rinsed with 0.01M phosphate-buffered saline (PBS) containing 0.3% Triton X-100, and incubated for 30 minutes at room temperature with 10% NGS followed by an overnight incubation at 4°C with the anti-TH antiserum (1:2000). Cultures were then washed 4 times with 0.01M PBS; rat cultures were then incubated overnight at 4°C with the anti-A2B5 antiserum (1:100) and washed 4 times with 0.01M PBS. TH-immunoreactive (TH-IR) neurons were detected by incubation in biotinylated goat anti-rabbit IgG (1:250), washed 4 times and incubated with avidin-biotin-peroxidase complex (0.6% v/v) for 1 hour at room temperature. Peroxidase was reacted with DAB (0.05% w/v) and hydrogen peroxide (0.003% v/v) to produce a brown reaction product. All rat cultures were then incubated for 1 hour at room temperature with biotinylated goat anti-mouse IgG (1:250), washed 4 times and incubated for 1 hour at room temperature with avidin-biotin-peroxidase complex to visualise A2B5 immunoreactivity. The

chromogen medium for this second reaction contained 0.02% (w/v) DAB with 0.006% (v/v) hydrogen peroxide in 0.05M Tris buffer, pH 7.5 and nickel ammonium sulfate (0.6% (w/v), Sigma) to produce a bluish-grey reaction product easily distinguishable from the dense brown of the previously stained TH-IR cells. Cell survival in rat cultures was determined microscopically by counting all TH-positive and A2B5-positive/TH-negative cells in 9 randomly selected 16x objective fields per dish. Maximum neurite extension, soma diameter and diameter of the major neurite were measured on 30 randomly chosen cells per dish, using the BioRad MRC-500 image analysis system (Fig. 16). All measurements were performed without knowledge of the drug treatment of the individual dishes being analyzed. Maximum neurite extension was defined, in constant arbitrary units, as the distance along the two longest neurites and across the cell soma, as traced by computer mouse. Somal diameter was measured along the straight line through the centre of the soma that yielded the maximum diameter; when this line extended into the base of a neurite, the boundary of the cell soma was defined as the point of inflection of the membrane. The maximum width of the major neurite was measured at least one soma diameter beyond this boundary (Fig. 15). In human cultures, all TH-IR cells on the entire culture surface were counted, and morphometric analysis was not performed.

For each of these measures, the means of the drug and control groups were compared using a one-way analysis of variance (ANOVA) with significance levels set at 5%. Where statistical significance was reached, Scheffé and Newman-Keuls

post-hoc tests were used to determine the source of the significance. Linear regression analysis was also performed, to assess the relationship between the dependent (measurements) and independent (treatment) variables.

5.3 Results

Tyrosine hydroxylase immunohistochemical staining in rats injected with 6-OHDA in the SNc revealed profound depletion of TH-immunoreactivity in the striatum ipsilateral to the 6-OHDA SN lesion (Fig. 14). Grafted TH-IR neurons with fibrous outgrowth were seen in both L-DOPA and saline treated animals (Fig. 15 and 16). *There were no significant differences between L-DOPA- and saline-treated groups for any parameters tested, including number of surviving TH-IR cells, graft-innervated volume and optical density.* The mean number ± S.E.M. of the surviving grafted TH-IR cells (Table 2) from every fifth 50μm section throughout the striatum in the saline-treated group was 747 ± 160 cells/graft, while the mean for the L-DOPAtreated group was 765 ± 153 cells/graft.

In order to estimate the average extent of neurite outgrowth from the graft into the host striatum, the optical density of the grafted and the unlesioned-contralateral striata in each animal were compared in two ways for both the saline and L-DOPA treated groups (Table 2). Values of mean overall optical density of grafted striata are presented as a percentage of mean overall contralateral striatal optical density in the same section. These values are 47.00 ± 8.63% for the drug treated group and 32.21 ± 4.67% for the saline treated group. Optical density

Figure 14. Low-magnification micrograph showing a dopamine depleted striatum (ST) ipsilateral to the 6-OHDA lesioned SN. Staining indicates TH-IR. Scale bar is 900 μm. (ST, striatum; cc, corpus callosum; ac, anterior commissure; ot, olfactory tubercle)

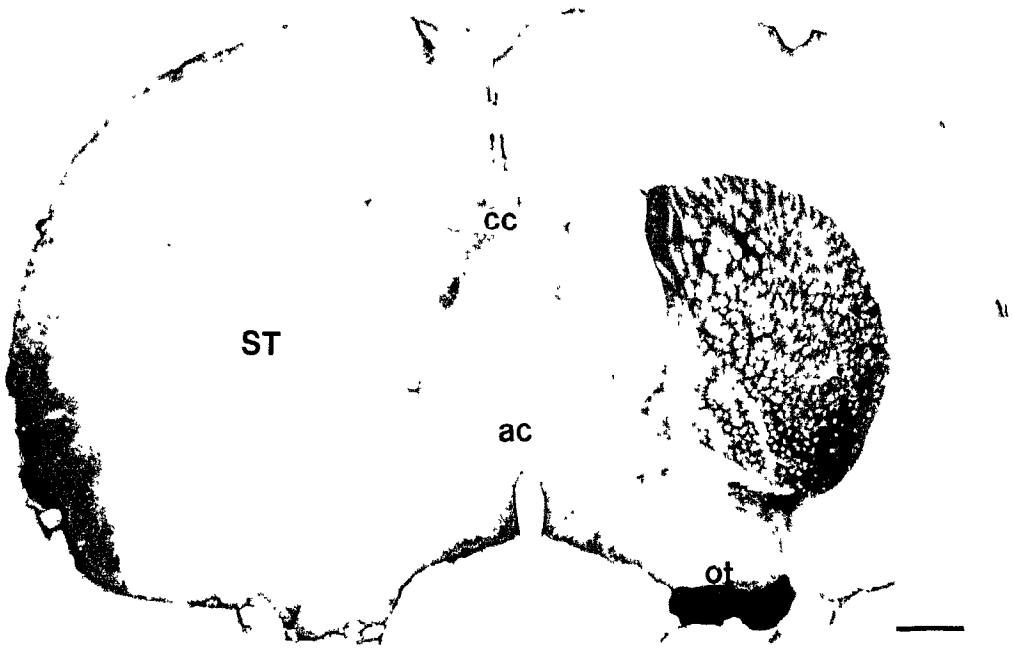


Figure 14

Figure 15. Low-magnification micrograph showing tyrosine hydroxylase (TH) immunohistochemical staining of striatum (ST) containing a dopaminergic graft ipsilateral to 6-OHDA lesion of the substantia nigra (compare staining of unlesioned contralateral striatum). Dotted line encloses area of graft-derived fibre outgrowth. CC, corpus callosum. Scale bar represents 500μm.

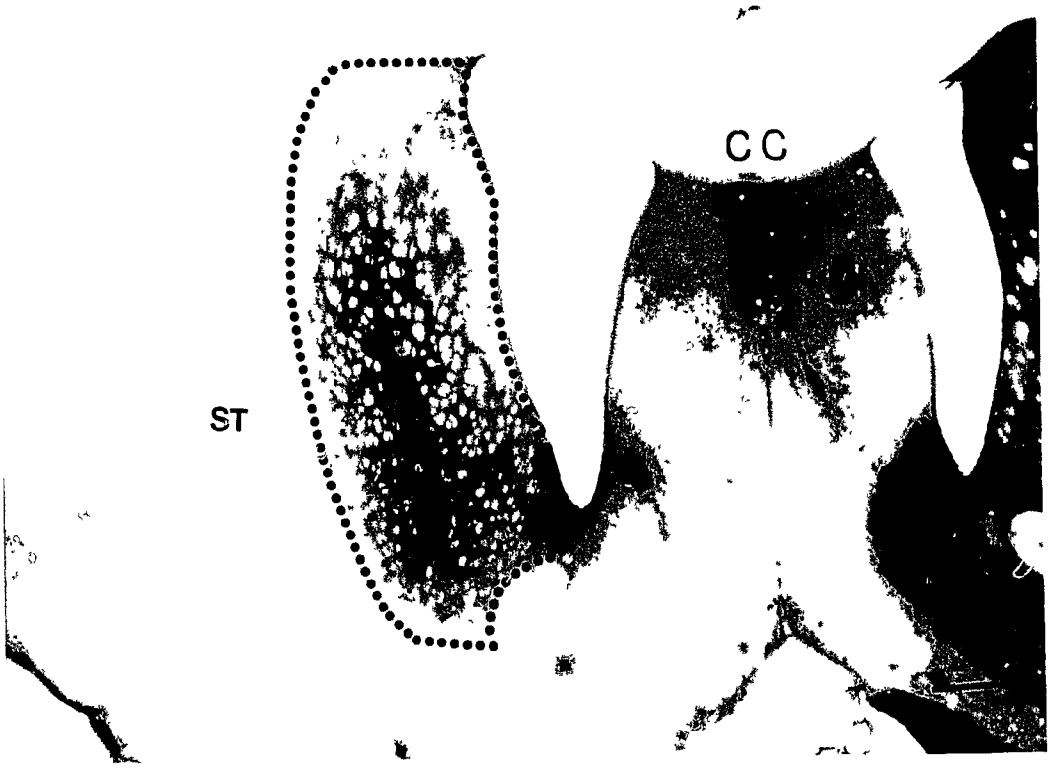


Figure 15

Figure 16. Higher-magnification micrograph showing TH-immunoreactive fibre outgrowth (arrowhead) from fetal ventral mesencephalic graft (G) into host striatum (ST). Arrows indicate grafted TH-IR neuronal cell bodies. Scale bar represents 80μm.

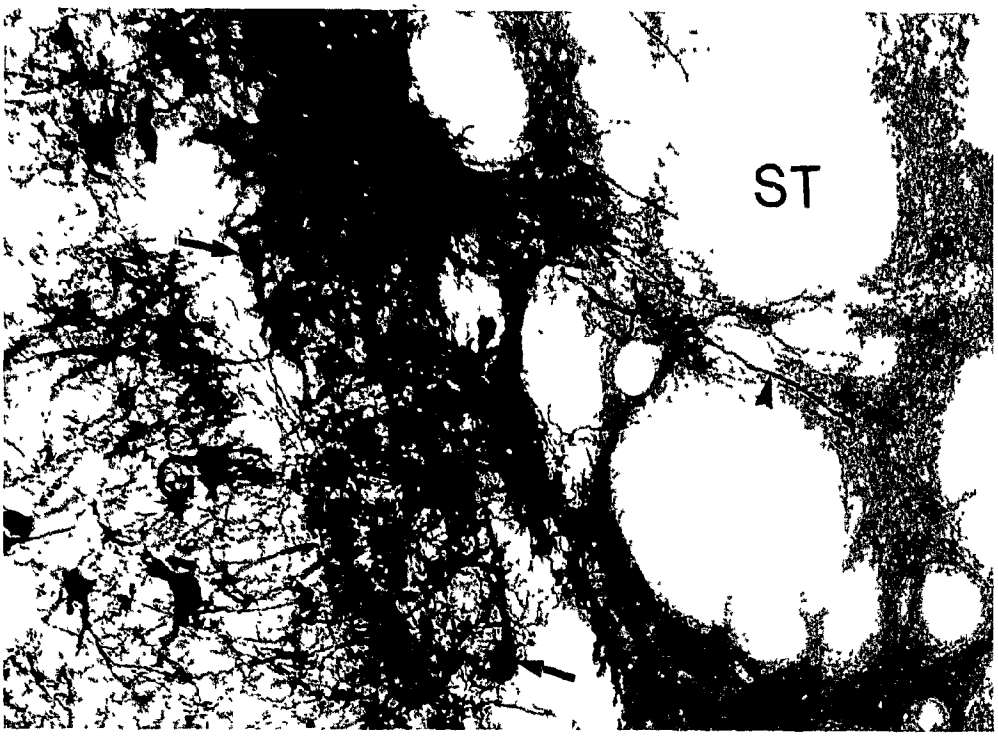


Figure 16

TABLE 2
Morphometric analysis of L-DOPA- and saline-treated ventral mesencephalic grafts.
Optical densities are expressed as percent of optical density of corresponding area of contralateral, unlesioned striatum in the same section. Full details of the measurements are given in the text. (From Chalmers and Fine, 1991)

	Number of TH-IR cells per graft (mean ± S.E.M.)	TH-IR optical density of entire grafted striatum (% ± S.E.M.)	TH-IR optical density of graft-innervated area (% ± S.E.M.)	Total graft-innervated volume (arbitrary units ± S.E.M.)
L-DOPA (n = 11)	762 ± 155	47.00 ± 8.63	85.18 ± 10.60	99.58 ± 22.37
Saline (n = 11)	747 ± 159	32.21 ± 4.67	72.01 ± 5.98	84.27 ± 17.10

compared with the equivalent area in the contralateral unlesioned striatum. These mean values are 85.18 ± 10.60% for the L-DOPA treated group and 72.01 ± 5.98% for the saline treated group. Graft-innervated volume, in arbitrary units, was compared between the two groups. The values were 99.58 ± 22.37 arbitrary units in the drug treated group and 84.27 ± 17.10 arbitrary units in the control group (Table 2).

was also determined for the cellular graft and its corresponding fibre outgrowth TH-IR cell number and neurite outgrowth *in vitro* were analyzed separately for bipolar or multipolar rodent cells; as no significant differences were found, results for these cell types were pooled. Measurements included number of TH-IR neurons (Fig. 19), number of A2B5-IR cells (Fig. 20), average maximum length of neurites (Fig. 18), average maximum width of major neurite and average soma diameter (Fig.17). The number of surviving TH-IR cells (Fig. 19) differed significantly from the pluronic-containing control only at the highest L-DOPA concentration, 1 x 10^{-5}M. In contrast, the number of A2B5-IR surviving cells (Fig. 20) did not differ significantly from controls at any L-DOPA concentration under study. The diameter of the cell soma was measured for each of 30 cells per cell culture dish ($n = 90$ for each drug dosage); there was no statistically significant difference among any of the control or drug groups (ANOVA) (Fig.21). No effect of L-DOPA upon maximum neurite extension of either multipolar and bipolar neurons at any dose was seen (Fig. 22). The maximum diameter of the major neurite was significantly different when comparing the means by ANOVA at different doses of L-DOPA. Scheffé's post-hoc analysis indicated this was due to small but significant increase in diameter, compared to controls, for the 5x10^{-6}M L-DOPA-treated cells only (Fig. 23).

Human ventral mesencephalic cultures were assayed for the total number of surviving TH-IR cells. Although this appeared to be a general downward trend of TH-IR cell number with varying L-DOPA concentrations, this trend was not

Figure 17. Differential interference contrast micrograph of rat ventral mesencephalic cells after ten days in culture, stained for A2B5 (*) and TH One large TH-IR neuron illustrates measurements of somal diameter (white arrow) and maximum neurite diameter (arrowheads). Unstained nuclei are visible amongst the TH-IR and A2B5-IR cells. Scale bar represents 20μm

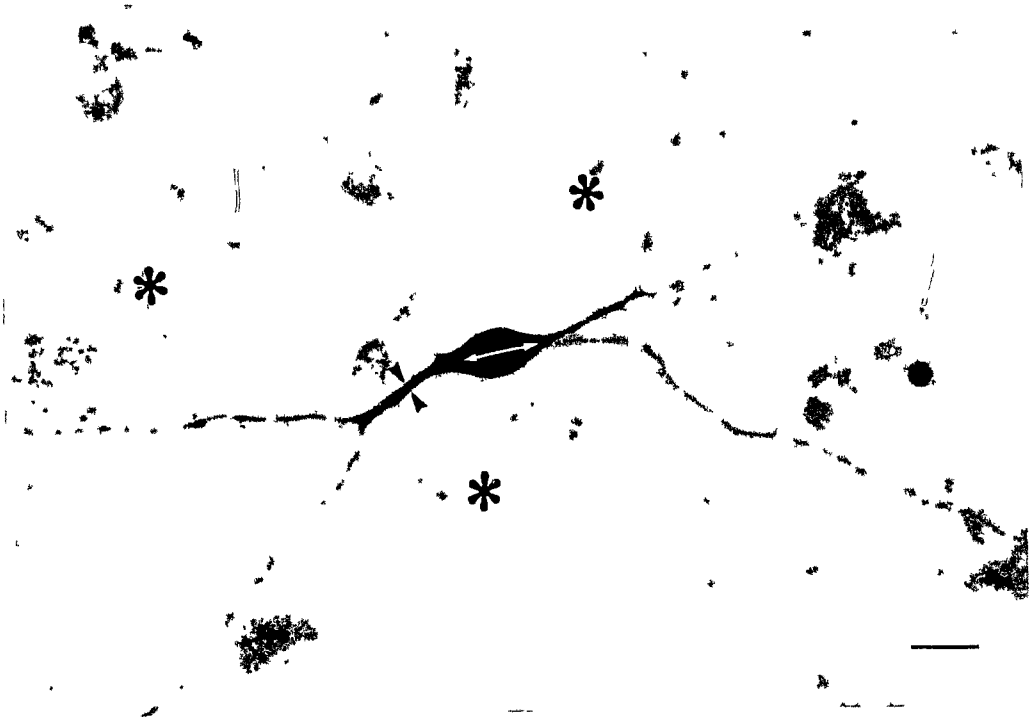


Figure 17

Figure 18. Two darkly-stained TH-IR rat neurons after ten days in culture. Arrows indicate the most proximal and distal aspects of the neurites extending from one cell body, illustrating measurements of maximum neurite outgrowth. For these measurements, neurites were traced interactively on a video monitor by computer mouse. Scale bar represents 50μm.

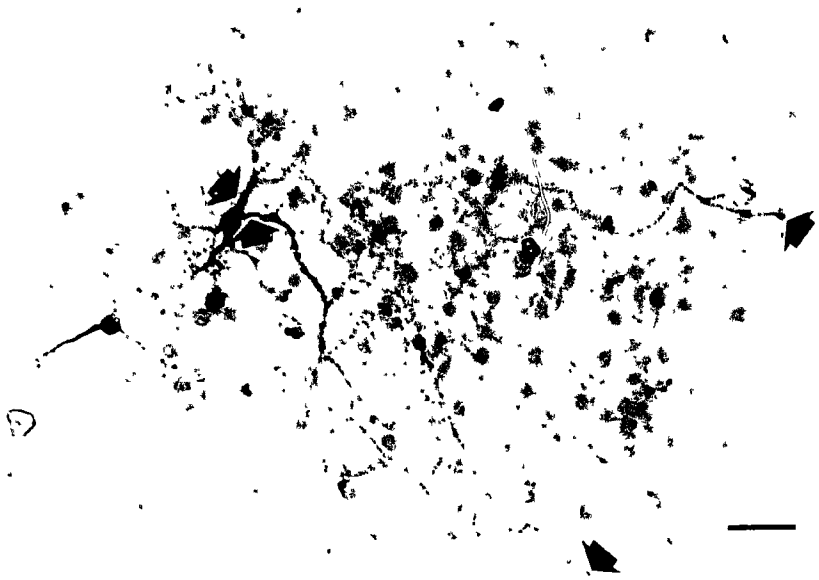


Figure 13

Figure 19. Graph showing the mean number ± S.E.M. of surviving rat TH-IR cells in 9 randomly chosen fields per culture vessel at different L-DOPA concentrations. Number of TH-IR cells did not differ significantly from controls at any L-DOPA concentration examined (ANOVA, $p < 0.05$). Bar indicates the number of TH-IR cells (± S.E.M.) in control medium.

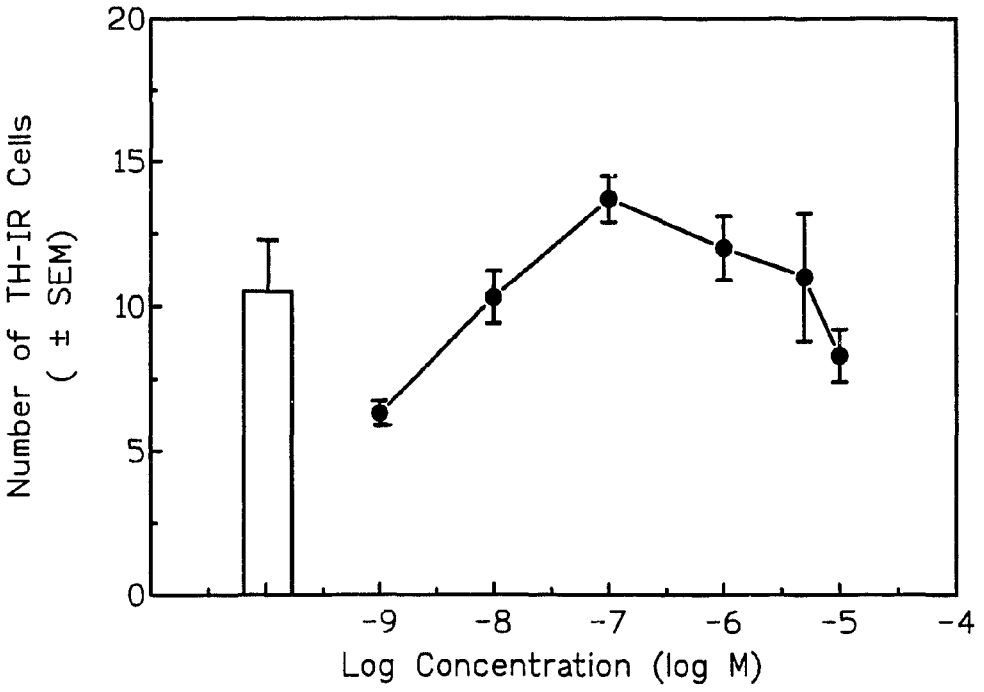

20
15
10
5
0
Number of TH-IR Cells
(± SEM)
-9
-8
-7
-6
-5
-4
Log Concentration (log M)

Figure 19

Figure 20. Graph showing the mean number ± S.E.M. of surviving A2B5-IR cells in the same 9 randomly chosen fields as in Fig. 18. The number of A2B5-IR cells did not differ significantly from controls at any L-DOPA concentration examined (ANOVA, $p<0.05$). Bar indicates the number of A2B5-IR cells (± S.E.M.) in control medium.

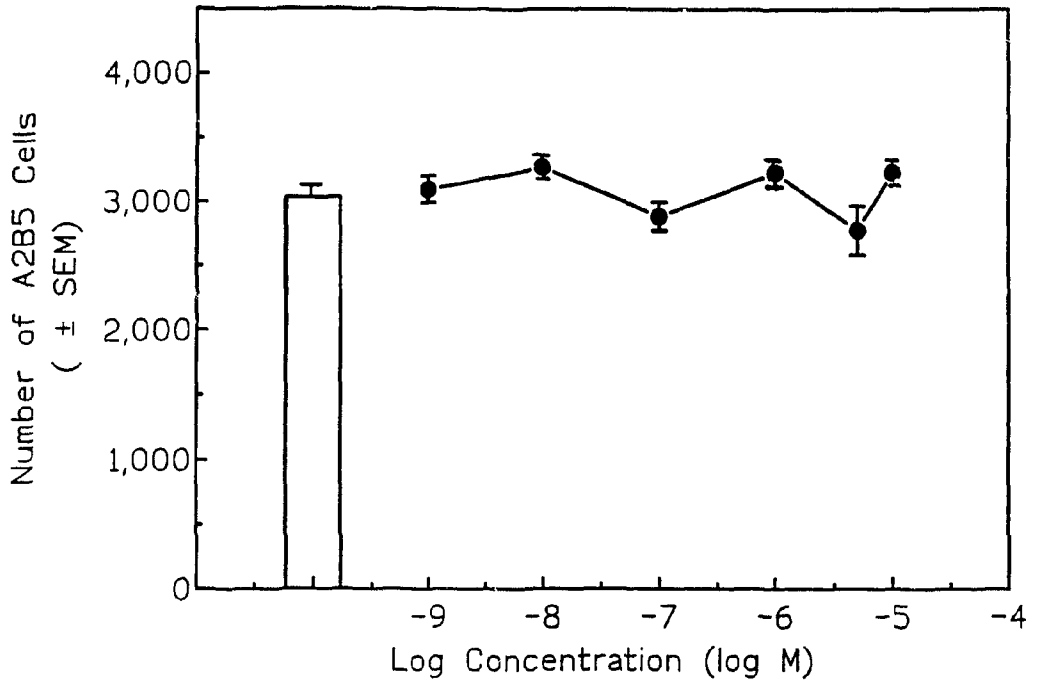

Number of A2B5 Cells
(± SEM)
4,000
3,000
2,000
1,000
0
-9
-8
-7
-6
-5
-4
Log Concentration (log M)

Figure 20

Figure 21. Graph showing the mean somal diameter ± S.E.M. of 30 randomly chosen rat TH-IR cells per culture vessel. Diameter did not differ significantly from control value (bar) at any L-DOPA concentration examined (ANOVA, $p < 0.05$).

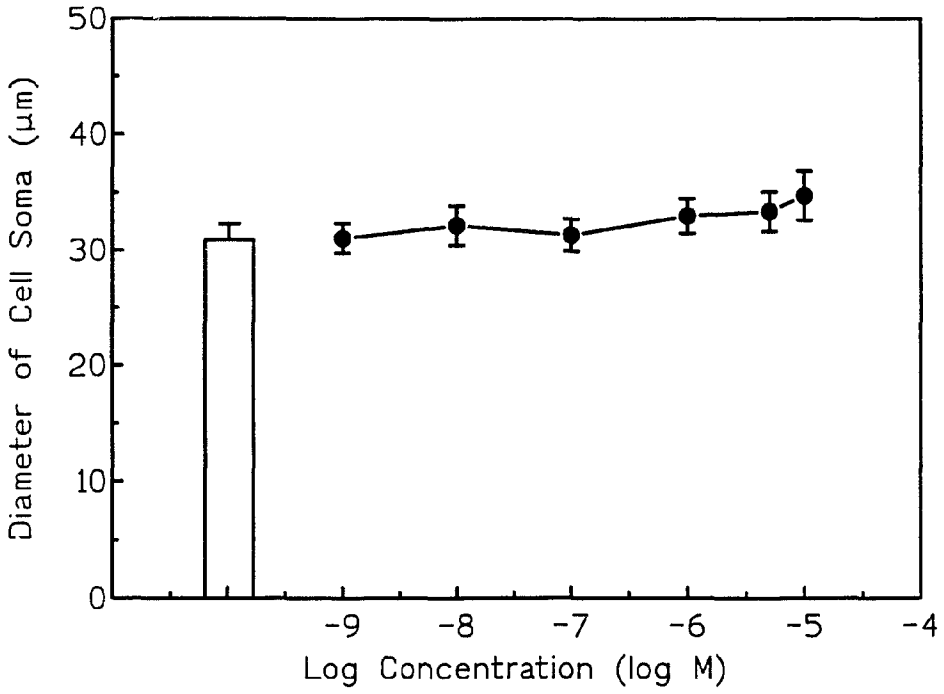

Diameter of Cell Soma (µm)
50
40
30
20
10
0
-9
-8
-7
-6
-5
-4
Log Concentration (log M)

Figure 21

Figure 22. Graph showing the mean major neurite extension ± S.E.M. from 30 randomly chosen rat TH-IR cells per culture vessel at varying L-DOPA concentrations. Extension measurements did not differ from the control value (bar) at any concentration examined (ANOVA, $p<0.05$).

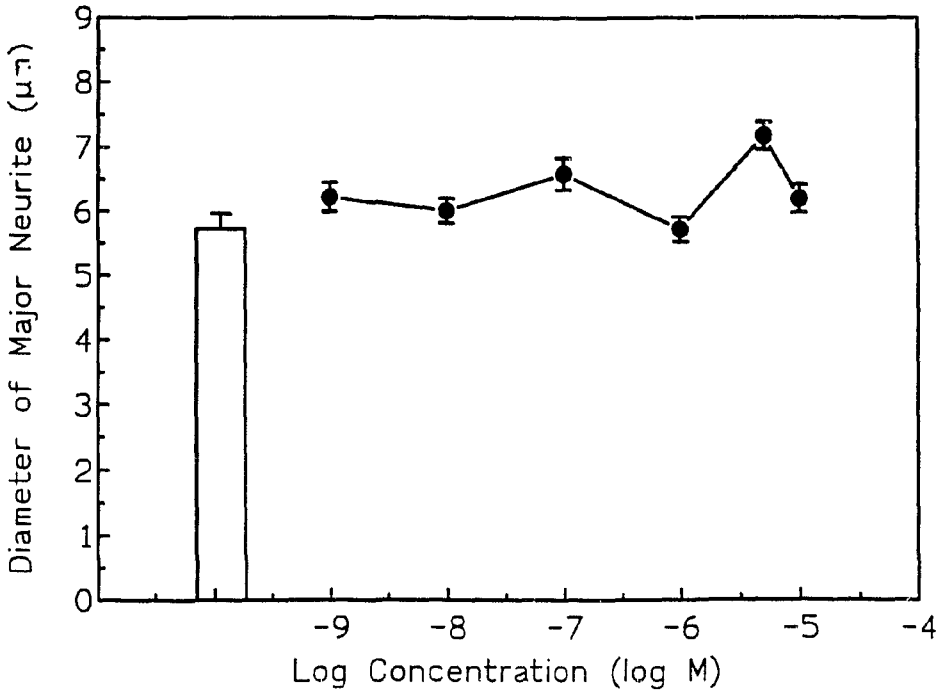

Diameter of Major Neurite (μm)
0
1
2
3
4
5
6
7
8
9
-9
-8
-7
-6
-5
-4
Log Concentration (log M)

Figure 22

Figure 23. Graph showing the mean major neurite width ± S.E.M. from 30 randomly chosen rat TH-IR cells per culture vessel. Width did not differ significantly from control value (bar) at any L-DOPA concentration examined (ANOVA, $p < 0.05$).

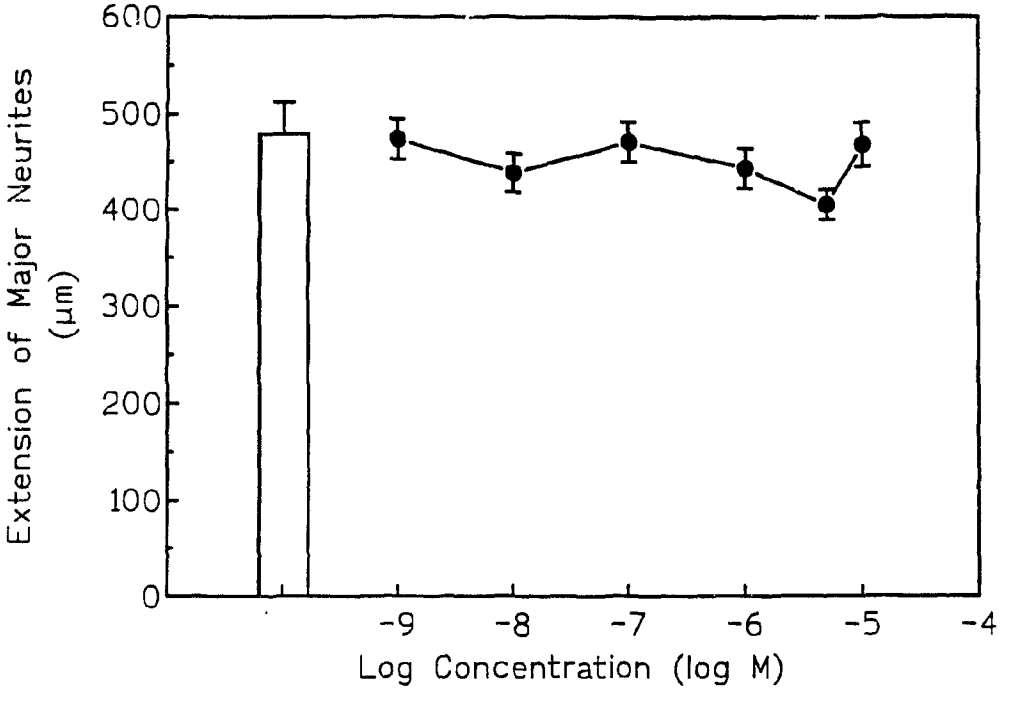

600
500
400
300
200
100
0
Extension of Major Neurites (µm)
-9
-8
-7
-6
-5
-4
Log Concentration (log M)

Figure 23

Figure 24. Graph showing the mean number ± S.E.M. of all TH-IR cells from the human ventral mesencephalon per well of a 24 well culture vessel. Number of TH-IR cells did not differ significantly from control (bar) at any L-DOPA concentration examined (ANOVA, $p < 0.05$).

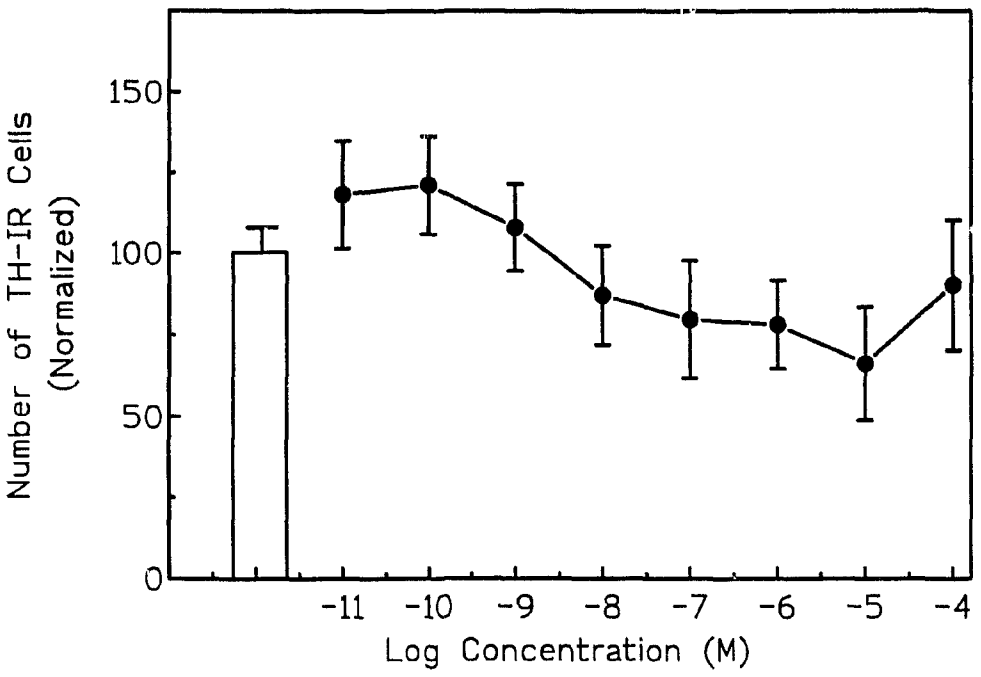

Number of TH-IR Cells
(Normalized)
150
100
50
0
-11
-10
-9
-8
-7
-6
-5
-4
Log Concentration (M)

Figure 24

significant (ANOVA), and the number of TH-IR cells did not differ significantly among any of the groups tested (ANOVA) (Fig. 24).

5.4 Discussion

Morphometric analysis of dopaminergic grafts following immunostaining revealed no significant difference in graft cell survival or fibre outgrowth after 8 weeks between animals treated with L-DOPA/carbidopa and similarly grafted animals treated with vehicle only. The range of doses used in this *in vivo* experiment is near the range of L-dopa doses generally used in PD therapy, 10 to 70 mg/kg per day (Sharpless and McCann, 1971; Ahlskog *et al.*, 1989). More rapid drug clearance in the rat could, however, invalidate direct comparison with PD patients; this problem could not be addressed in our *in vivo* study. It is also possible that more prolonged exposure of dopaminergic neurons to these high levels of L-DOPA would have deleterious effects not observed here. Optical density measurements combine cell density and/or fibre outgrowth with intensity of staining, in a single lumped parameter. We made no attempt to isolate these elements in our analysis of *in vivo* effects, other than to examine cellular, graft-innervated and total striatal areas separately; in no case did significant treatment effects emerge.

To control the L-DOPA levels more precisely, fetal rat and human dopaminergic ventral mesencephalic cells were exposed to the drug *in vitro* over a range of L-DOPA concentrations, from $1x10^{-9}$ to $1x10^{-4}$M, which encompasses

the ordinary range of cerebrospinal fluid L-dopa concentrations in PD therapy of $5x10^{-8}$ to $2x10^{-6}$M (Ahlskog *et al.*, 1989). No carbidopa was added to these *in vitro* preparations since the grafted cells would presumably be in contact with it for only the brief period of postsurgical blood-brain barrier breakdown. The number of surviving TH-IR cells differed significantly from control only at the highest L-DOPA dose tested in rat, a dose ten times higher than the upper limit of the therapeutic range reported by Ahlskog *et al.* (1989). There was no preferential survival of rat bipolar versus multipolar TH-IR neurons at this (or any) L-DOPA concentration. Soma diameter, maximum neurite extension and maximum neurite diameter were measured in order to investigate possible sublethal drug effects. Only maximum neurite diameter displayed a significant effect: an increase versus control at a dose of $5x10^{-6}$M; linear regression analysis, however, revealed a poor correlation between neurite diameter and L-DOPA dose. No effect of L-DOPA on survival of A2B5-IR cells was seen at any dose tested, indicating that the reduction in TH-IR cell number at $1x10^{-5}$M L-DOPA reflects selective dopaminergic toxicity. The number of human TH-IR surviving cells did not differ from control at any L-DOPA dose tested. This was the only parameter measured for these cells because preliminary observations of the cells at different L-DOPA doses did not warrant further examination. The findings of non-specific differences seen in the fetal rat cultures may be of relevance to the natural loss of catecholaminergic neurons throughout life (McGeer and McGeer, 1976) or to the depletion of dopaminergic neurons in PD (Hornykiewicz, 1966). An increase in dopamine due to exogenous

L-DOPA metabolism might, for example, lead to increased production of reactive semiquinones and other free radicals (Graham *et al.*, 1978; Smith *et al.*, 1994) which may be toxic to ventral mesencephalic dopaminergic neurons. The beneficial effects of selegiline (Cohen and Spina, 1989; Roy and Bédard, 1993) lend credence to this hypothesis. However, the observation of toxic effects of L-DOPA only at doses beyond the therapeutic range and more importantly, the fact that no ill effects were seen in relation to fetal human *in vitro* studies does not support the hypothesis that such toxicity can account for poor dopaminergic graft survival and function in PD patients and supports the findings of others illustrating lack of toxic effect of L-DOPA on ventral mesencephalic cells (Perry *et al.*, 1984; Hefti *et al.*, 1981; Carvey *et al.*, 1991) and grafts (Blunt *et al.*, 1993). This same reasoning implies that L-DOPA therapy for such patients need not to be discontinued following fetal dopaminergic neuronal transplantation.

Nevertheless, important caveats remain. In contrast to these findings, others have reported that nominally higher-dose L-DOPA/carbidopa injections impair dopaminergic graft and cell survival (Olney *et al.*, 1990; Steece-Collier *et al.*, 1990, Mytilineou *et al.*, 1993). Since their duration of treatment was substantially shorter than ours, their observations might reflect higher-dose toxicity beyond a realistic therapeutic range, or a sublethal slowing of dopaminergic cell development rather than direct cell loss. Furthermore, it is known that dopaminergic neurons in the rat are significantly less vulnerable to 1-phenyl-4-methyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity (Sirinathsinhji *et al.*, 1988)

than are humans where MPTP produces a syndrome closely resembling idiopathic PD (Langston *et al.*, 1984) and the LD_{50} of L-DOPA in rats is considerably higher than in other species such as rabbits.

6.0 General Discussion and Future Research

The overall objective of the research described here was to identify factors affecting the survival, growth and development of CNS neurons in the fetal rat and human. In idiopathic Parkinson's disease, the factor responsible for the death of the striatal-projecting dopaminergic cells in the substantia nigra pars compacta is unknown. The motor dysfunction associated with PD results mainly from an abnormal reduction in number of these TH-IR cells, although cholinergic and noradrenergic neurons from other brain areas may be affected (Agid *et al.*, 1989). Pharmacological therapy for PD involves administration of the dopamine precursor, L-DOPA, to increase brain levels of dopamine which declines due to this massive loss of TH-IR cells. This therapy, however, often has undesirable side effects, asnd loses effectiveness over time. Other therapies include anticholinergic drug therapy, in an attempt to compensate the imbalance between acetylcholine and dopamine in the striatum that results from nigral degeneration, and selegiline at dosages sufficient to inhibit monoamine oxidase B (Roy and Bedard, 1993) which is active in the catabolism of dopamine which may produce highly reactive oxidative free radicals. No drug therapy or surgical procedure, however, has yet been described that arrests the disease process.

This thesis work began as an examination of L-DOPA and its potential to exert toxic effects on TH-IR cells but developed into a more general investigation of agents that affect the death and survival of these cells. This led to the

examination of possible effects of target cells through lesion and graft studies and actions of specific growth factors. Growth factors, particularly EGF and IGF-I, have been shown to be necessary for the development of human progenitor cells from human fetal forebrain. These cells have been partially characterized and have been shown to differentiate into neurons and glia comprised of a variety of phenotypes including TH-IR cells. With further examination and manipulation of the culture conditions, it might be possible to direct the differentiation to yield a purely dopaminergic population. Such a population would be useful for cell replacement therapy which at this time is complicated due to the use of tissue derived from therapeutically aborted fetuses or transformed cells which may form tumours *in vivo*. There are a number of populations of progenitor or stem cells in the adult CNS including cells from the subventricular zone and the dentate gyrus of the hippocampus which are likely strictly regulated by cellular interactions, growth factors or other peptides. It is therefore possible that the population of progenitor cells described here is a normally occuring population which is allowed to grow uninhibited in culture making possible the manipulation for differentiation. Future work may address this directed differentiation and the study of the resulting populations of postmitotic neurons.

I have shown that lesioning the striatal target of SN neurons leads to a retrograde loss of nigral TH-IR cells and that this loss may be reduced by partial reconstruction of the lesioned striatum by grafted fetal striatal primordia. A decrease in volume of the SNr was noted after lesioning, which was unaffected by

grafting. This decrease could result from extensive fibre loss in the SNr due to the excitotoxic lesion induced death of GABAergic projection neurons in the striatum rather than SNr cell death due to loss of trophic support. TH-IR cell size, however, which has been reported to decrease after striatal lesioning (Lundberg *et al.*, 1993), increased slightly but not significantly. This suggests the possibile existence of a population of surviving TH-IR neurons that sprout to reform striatal connections within the lesioned target in response to the lesion-induced death of other TH-IR neurons. Lundberg *et al.* measured retrogradely-labled, striatal projecting cells but did not carry out TH-IR staining of these cells. Their observation of a decrease in these cells' size may include degenerating cells that no longer express TH (Blanchard *et al.*, 1994) or possibly non-dopaminergic projecting neurons (Gerfen *et al.*, 1985).

I examined specific growth factors for their *in vitro* effects on human TH-IR ventral mesencephalic cell number. bFGF with heparin exerted the greatest effect, followed by bFGF alone and BDNF. The IGFs exerted no significant effect on human ventral mesencephalic TH-IR cell number in this study. Whether the observed effects resulted from increased survival or proliferation was not addressed and is a topic for further investigation.

I have established that CNS progenitor cells can be isolated from fetal human forebrain and made to propagate, for a presumably finite period of time which we were not able to determine in our culture system, and differentiate in the presence of specific culture medium and growth factor control *in vitro*. It may

be possible, with further investigation, to steer the differentiation of these cells by carefully manipulating culture conditions, resulting in a preferential differentiation of the progenitor cells into specific neuronal or glial phenotypes. For example, by depleting tyrosine from the growth medium, it may be possible to select for dopaminergic cells since these cells can utilize phenylalanine instead (D. Morassutti, personal communication). Similarly, the addition of specific growth factors may support the survival of specific cells. The development of such methods would be valuable for the understanding the control of differentiation, and possibly also for clinical transplant procedures where such cultured cells could provide a viable alternative to primary human fetal tissue in which TH-IR cells are a variable minority. Dopamine-producing cell lines from transgenic animals have been described (Suri *et al.*, 1993), and their use in clinical transplantation has been suggested. However, the possibilities of tumour formation and graft rejection pose serious obstacles to their use. Human neurons derived from a propagating populations of untransformed progenitor cells could provide a superior alternative as a source of clinical graft material. Although it has been shown that dopaminergic neurons from nuclei other than the SNc do not provide appropriate innervation of the target striatum (Abrous *et al.*, 1988; Bjorklund *et al.*, 1983; Dunnet *et al.*, 1989), it may be possible with further characterization to manipulate surface antigens or molecular mechanisms related to cell recognition (which at this time are unknown) to enhance integration. Further investigation into the characteristics and differentiation potentials of these

cells will be necessary however to reach this stage of investigation.

Grafting of dopaminergic neurons from the fetal substantia nigra into the deafferented striatum of Parkinsonian patients has been attempted in many centres with varying successes (Lindvall *et al.*, 1992; Freed, 1993). Unfortunately, at this time, a standard evaluation procedure is not in place to directly compare results from the various studies but the successes demonstrated are encouraging and warrant further investigation (Langston *et al.*, 1992). Through the use of grafts, much has been learned about neural plasticity. Clinical grafting in PD patients has resulted in some improvement in disease symptoms but no symptom has been improved to a normal level and therefore the major focus is directed towards improving the motor performance of affected individuals.

The source of graft tissue is of utmost importance. Adrenal chromaffin cells grafted into the deafferented striatum of the rat yielded a reduction in rotational behaviour to such a degree (Freed *et al.*, 1981) that the procedure was extended to human PD therapy, but with disappointing results (Freed *et al.*, 1990). More recently, human fetal dopaminergic material has become the transplantation tissue of interest because of its ability to survive and grow in the adult deafferented target and perhaps most importantly, create and maintain functional and appropriate synaptic connections with the host target cells (Sawle *et al.*, 1992). Some symptoms such as rigidity and bradykinesia are attenuated and L-DOPA dosages decreased but others such as tremor are unaffected by fetal dopaminergic cell grafting.

It must always be appreciated that the origin of the transplanted tissue is that of the fetal nervous system which normally resides in a drastically different environment from that of the adult. It may therefore be appropriate to identify factors that enhance the survival of these cells but are not necessarily present in the adult target for the purpose of providing the appropriate factors to the cells upon transplantation and possibly to be maintained for some unspecified time. I have identified two such factors that enhance human fetal dopaminergic cell survival *in vitro*, BDNF and bFGF.

The dopaminergic metabolite, L-DOPA, has been shown in this thesis not to affect the *in vitro* or *in vivo* survival of rat mesencephalic TH-IR cells or the *in vitro* survival of human ventral mesencephalic TH-IR cells. However with regard to human TH-IR cells, a trend towards a mild toxic effect was noted that did not reach significance. This issue has been controversial based on previously published observations (Steece-Collier *et al.*, 1990; Chalmers and Fine, 1991; Yurek *et al.*, 1991; Blunt *et al.*, 1992; Mena *et al.*, 1992; Paino *et al.*, 1992; Mytilineou *et al.*, 1993; Pardo *et al.*, 1995). In my examination of this interaction, L-DOPA concentrations were tested over normal therapeutic ranges (Ahlskog *et al.*, 1989), whereas the toxic effects described in the aforementioned studies were obtained in some cases only at higher levels. This issue is of importance with respect to clinical trials of fetal human dopaminergic transplants into the striatum of patients suffering from PD (Lindvall., 1991; Freed *et al.*, 1990; Lindvall *et al.*, 1994). The results described here indicate that withdrawal of drug

therapy prior to transplantation is not necessary.

The cause of the accelerated loss of TH-IR cells in PD is not known. Growth factor levels have been shown to decrease in PD but whether this is causal or incidental is not known. bFGF and BDNF have previously been shown to enhance the survival of human fetal dopaminergic cells *in vitro* (Silani *et al.*, 1994; Zhou *et al.*, 1995). bFGF has been shown to protect dopaminergic neurons from the neurotoxic effects of MPTP in mice (Otto and Unsicker, 1990). It is possible that bFGF affects the oxidative free radical formation thereby reducing this naturally occurring cell damage. bFGF treatment e[illegible]ly in PD may slow the death of dopaminergic cells and therefore the progression of the disease. Data in this thesis suggest that heparin can enhance the effect of bFGF by more than 3 fold. These observations are of relevance to human fetal transplantation, in that the addition of growth factors to the initial grafted cell suspension, and/or the subsequent delivery to the brain, may enhance the survival, anatomical integration and function of the grafts. Furthermore, it is an important possibility that depletion of growth factors may contribute to the degeneration of specific cell populations in the progression of such neurological diseases as Parkinson's, Alzheimer's and Huntington's diseases and that supply of the missing growth factors may slow the disease progression.

In conclusion, the results presented here suggest that a multitude of factors affect the growth and development of human CNS cells. From the developing human brain, it is possible to isolate a population of progenitor cells which upon

differentiation manifest a variety of neuronal and glial phenotypes including tyrosine hydroxylase expression. The target cells in the rat striatum exert profound trophic influences on projection neurons from the SN. BDNF, bFGF and heparin supplemented bFGF enhance the survival of human fetal dopaminergic neurons *in vitro* and L-dopa is not toxic to these cells in either rat or human. Further investigation can be expected to increase our understanding of the potential of dopaminergic growth factors and human fetal progenitor cells both of which may lead to new strategies in the neural cell transplantation and the treatment of Parkinson's disease.

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