The role of the dopaminergic neurotrophin growth/differentiation factor-5 in the developing rat ventral midbrain

By

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And the end of our exploring

Will be to arrive where we started

And know the place for the first time.

T. S. Eliot (1942)

Preface

All work presented in this thesis is original and entirely my own. The work was carried out under the supervision of Dr. Aideen Sullivan between October 2000 and October 2004 in the Department of Anatomy, University College, Cork, Ireland. This dissertation has not been submitted in whole or in part for any other degree, diploma or qualification at any other University.

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Publications arising from this work

Abstracts

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Papers

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1.0 ABSTRACT

Growth differentiation factor-5 (GDF-5) is a member of the transforming growth factor-β superfamily, a family of proteins that play diverse roles in many aspects of cell growth, proliferation and differentiation. GDF-5 has also been shown to be a trophic factor for embryonic midbrain dopaminergic neurons *in vitro* (Krieglstein et al. 1995) and after transplantation to adult rats *in vivo* (Sullivan et al. 1998). GDF-5 has also been shown to have neuroprotective and neurorestorative effects on adult dopaminergic neurons in the substantia nigra in animal models of Parkinson's disease (Sullivan et al. 1997, 1999; Hurley et al. 2004). This experimental evidence has lead to GDF-5 being proposed as a neurotrophic factor with potential for use in the treatment of Parkinson's disease. However, it is not know if GDF-5 is expressed in the brain and whether it plays a role in dopaminergic neuron development. The experiments presented here aim to address these questions. To that end this thesis is divided into five separate studies each addressing a particular question associated with GDF-5 and its expression patterns and roles during the development of the rat midbrain.

Expression of the GDF-5 in the developing rat ventral mesencephalon (VM) was found to begin at E12 and peak on E14, the day that dopaminergic neurons undergo terminal differentiation. In the adult rat, GDF-5 was found to be restricted to heart and brain, being expressed in many areas of the brain, including striatum and midbrain. This indicated a role for GDF-5 in the development and maintenance of dopaminergic neurons.

The appropriate receptors for GDF-5 (BMPR-II and BMPR-Ib) were found to be expressed at high levels in the rat VM at E14 and BMPR-II expression was demonstrated on dopaminergic neurons in the E13 mouse VM. GDF-5 resulted in a three-fold increase in the numbers of dopaminergic neurons in cultures of E14 rat VM, without affecting the numbers of neurones or total cells. GDF-5 was found to increase

the proportion of neurons that were dopaminergic. The numbers of Nurr1-positive cells were not affected by GDF-5 treatment, but GDF-5 did increase the numbers of Nurr1-positive cells that expressed tyrosine hydroxylase (TH). Taken together this data indicated that GDF-5 increases the conversion of Nurr1-positive, TH-negative cells to Nurr1-positive, TH-positive cells. In GDF-5 treated cultures, total neurite length, neurite arborisation and somal area of dopaminergic were all significantly increased compared to control cultures. Thus this study showed that GDF-5 increased the numbers and morphological differentiation of VM dopaminergic neurones *in vitro*.

In order to examine if GDF-5 could induce a dopaminergic phenotype in neural progenitor cells, neurosphere cultures prepared from embryonic rat VM were established. The effect of the gestational age of the donor VM on the proportion of cell types generated from neurospheres from E12, E13 and E14 VM was examined. Dopaminergic neurons could only be generated from neurospheres which were prepared from E12 VM. Thus in subsequent studies the effect of GDF-5 on dopaminergic induction was examined in progentior cell cultures prepared from the E12 rat VM.

In primary cultures of E12 rat VM, GDF-5 increased the numbers of TH-positive cells without affecting the proliferation or survival of these cells. In cultures of expanded neural progenitor cells from the E12 rat VM, GDF-5 increased the expression of Nurr1 and TH, an action that was dependent on signalling through the BMPR-Ib receptor.

Taken together, these experiments provide evidence that GDF-5 is expressed in the developing rat VM, is involved in both the induction of a dopaminergic phenotype in cells of the VM and in the subsequent morphological development of these dopaminergic neurons.

2.0 ABBREVIATIONS

AA	ascorbic acid
Act	activin
ActR	activin receptor
AM	adrenal medulla
ANOVA	analysis of variance
APS	ammonium persulphate
BDNF	brain derived neurotrophic factor
BMP	bone morphogenetic protein
BMPR	bone morphogenetic protein
BMSC	bone marrow stromal cells
bp	brachypodism
Brd-U	5-Bromo-2-deoxyuridine
BSA	bovine serum albumin
ca	constitutively active
CB	carotid body
CHO	chinese hamster ovary
Co-smad	common mediator smad
CREB	cAMP responsive element binding
CBP	cAMP responsive element binding-binding protein
DIV	days in vitro
DMEM	dulbecco's minimal essential medium
DVR	Dpp-Vgr1-related
E	embryonic day
EGF	epidermal growth factor
EMEM	eagle minimum essential medium
FCS	foetal calf serum
FGF-2	fibroblast growth factor
GABA	gamma-amino-butyric acid

GDF	growth differentiation factor
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GPe	Globis pallidus external segment
GPi	Globis pallidus internal segment
h	hour
HBSS	Hank's balanced salts solution
HLH	helix-loop-helix
HRP	horseradish peroxidase
I-smad	inhibitory smad
IL	interleukin
LIF	leukemia inhibitory factor
M	molarity
MAO	monoamine oxidase
MBP	myelin basic protein
MH	mad homology
MIC	macrophage inhibiting cytokine
min	minute
MPP^+	n-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	magnetic resonance imaging
N	number
NGS	normal goat serum
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NPC	neural progenitor cells
NSC	neural stem cells
6-OHDA	6-hydroxydopamine
p	probability
P	postnatal day

PD	parkinson’s disease
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with Tween-20
PVDF	polyvinylidene difluoride
R-smad	receptor activated smad
rh	recombinant human
RPE	retinal pigmented epithelium
SD	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error
Shh	sonic hedgehog
SN	substantia nigra
SOD	superoxide dismutase
STN	subthalamic nucleus
SVZ	subventricular zone
TGF-β	transforming growth factor-β
TH	tyrosine hydroxylase
VM	ventral mesencephalon
VZ	ventricular zone

3.0 INTRODUCTION

3.1 Parkinsons Disease

First described by James Parkinson in 1817, the disease has since come to bear his name (Parkinson 2002). Parkinson's disease (PD) is a slowly progressing neurodegenerative disorder, the pathological hallmark of which involves degeneration of dopaminergic neurons in the substantia nigra (SN) of the midbrain (A9 group of neurons) (Jellinger et al. 1991). The disease has been reported to affect 0.1% of the total population and approximately 1% of the population over 65 years of age (for review see Marsden 1994). The degeneration of these neurons leads to a loss of dopaminergic innervation of the striatum and consequently reduced dopamine levels in the striatum (Llyod and Hornykiewicz 1970). The SN is part of a group of subcortical nuclei called the basal ganglia, comprising the striatum (or caudate/putamen), the globus pallidus (internal (GPi) and external (GPe) segments), the subthalmic nucleus (STN), the thalamus, the pedunculopontine nucleus and the SN (Alexander and Delong 1990). The basal ganglia can be viewed as part of a larger cortico-basal gangli-thalmo-cortical pathway that is responsible for mediating voluntary movement (Alexander and Delong 1990). In PD, the loss of dopaminergic neurons in the SN results in overactivity of the output nuclei of the basal ganglia, the STN and the GPi. As these structures use the inhibitory neurotransmitter gamma-amino-butyric acid (GABA), the overactivity of these nuclei leads to excessive inhibition of thalamic nuclei, which leads to inhibition of the cortical motor system, resulting in the three classical symptoms of PD, bradykinesia (slowness of movement), akinesia (difficulty to initiate movement) and resting tremor (for reviews see Alexander and Delong 1990, Bonnet and Houeto 1999). It is believed that inhibitory descending projections to the brainstem nuclei, namely the pedunculopontine nucleus,

the superior colliculus and the mesopontine tegmentum is responsible for abnormalities in posture and gait (Parent and Cicchetti 1998).

3.1.1 Etiology

Although the pathology of PD has been well characterised, the etiology of this disease is unknown. However, a number of factors have been shown to increase the risk of developing PD. These include exposure to well water, herbicides, pesticides, industrial chemicals, wood pulp mills farming and rural environments (for review see Tanner and Langston 1990). However, no specific environmental or chemical agents can be directly attributed as the causative agent for PD. The argument that exogenous toxins are responsible for PD was strengthened by the discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is a byproduct of the chemical synthesis of an analogue of the opiate, meperidine (Langston et al. 1983). Drug addicts who took opiate preparations containing MPTP developed a condition that strikingly resembled PD at the clinical and pathological level (Langston et al. 1983). In recent years it has emerged that MPTP crosses the blood brain barrier and is converted to N-methyl-4-phenylpyridine (MPP+) by monoamine oxidase B (MAO-B) in astrocytes (Singer et al. 1987). It is selectively taken up by dopamine neurons in the SN, where it causes a defect in complex I of the mitochondrial respiratory chain, which is similar to that found in PD (Nicklas et al. 1985, Singer et al. 1987, Schapira et al. 1990). MPP+ has been shown to induce apoptotic neuronal death in cultures of the embryonic rat midbrain (Mytilineou et al. 1985) and in the human dopaminergic cell line SH-SY5Y (Itano and Nomura 1995). As apoptosis has been detected in the SN of PD patients (Mochizuki et al. 1996, Anglade et al. 1997), it is now thought that MPP+ induced parkinsonism results from apoptosis of dopaminergic neurons of the SN through a blocakage of complex I of the mitochondrial respiratory chain (Fall and Bennett 1999). These

findings suggest that certain environmental toxins may mimic the action of MPTP/MPP+ and be responsible for at least some cases of idiopathetic PD (for review see Di Monte et al. 2002, for detailed review of mechanisms of MPTP neurotoxicity see Przedborski and Vila 2001).

3.1.2 Genetic factors

Although the majority of PD cases are idiopathetic, it has emerged that approximately 5-10% of all cases are genetic in origin (for review see Pankratz and Foroud 2004). A large number of DNA polymorphisms have been studied in the search for a genetic risk factor for PD. However, polymorphisms in many genes found in some PD cases have been shown not to confer an increased risk of PD, while others do confer an increased risk (for a complete list, see Mizuno et al. 1999, Pankratz and Fourod 2004). Because a defect in complex I of the mitochondrial respiratory chain has been found in PD (Nicklas et al. 1985), extensive searching of the mitochondrial genome for mutations associated with the disease was performed. A polymorphorism in manganese-containing superoxide dismutase was found to be a risk factor for PD (for review see Mizuno et al. 1999, Pankratz and Fourod 2004), but no disease-specific mutation in the mitochondrial genome has been identified. Although a large number of families have been identified in which members in different generations suffer from PD (for review see Duvoisin et al. 1998, Mizuno et al. 1999, Pankratz and Fourod 2004), disease-causing mutations in specific genes of known function have been difficult to identify. A few exceptions have been the identification of mutations in genes encoding α-synuclein (Polymeropoulos et al. 1997), parkin (Kitada et al. 1998), UCH-L1 (Leroy et al 1998) and tau (Hutton et al. 1998), which have been shown to be responsible for PD in certain families (for review see Olanow and Tatton 1999, Skipper and Farrer 2002).

3.2 Pathogenesis of Parkinson's disease

While in most cases the causes of idiopathetic PD are unknown, a number of different mechanisms have been shown to be involved in the pathogenesis of PD. These include oxidative stress, mitochondrial dysfunction and excitotoxicity.

3.2.1 Oxidative stress

Oxidative stress was proposed as a major factor in the pathogenesis of PD after it became known that the metabolism of dopamine can generate hydrogen peroxide. Hydrogen peroxidase can interact with iron, generating reactive oxygen species and lead to oxidative damage and cell death (for review see Olanow and Tatton 1999). Free hydrogen peroxidase is normally cleared by reduced glutathione. A role for oxidative stress in the pathogenesis of PD was strengthened when it was shown that free iron levels are increased in the SN of PD patients (for review see Olanow and Youdim 1996). Furthermore when infused into the SN of rodents it caused progressive degeneration of SN dopaminergic neurons (Sengstock et al. 1993). Reduced glutathione levels and evidence of oxidative damage have also been found in the SN of PD patients (for review see Olanow and Tatton 1999), suggesting that oxidative damage is a contributing factor in the pathogenesis of PD. Whether is it the primary causative agent or it occurs secondary to an alternate etiology remains to be determined.

3.2.2 Mitochondrial dysfunction

A decrease in complex I activity of the mitochondrial respiratory chain has been found in the SN of PD patients (Nicklas et al. 1985, Schapira et al. 1990). It has been shown that blockage of complex 1 of the mitochondrial electron transport chain by MPP+ leads to depletion of cellular ATP levels, ultimately leading to apoptosis (Song et al. 1997). It has also been proposed that inhibition of the mitochondrial electron transport chain by

MPP+ leads to the leakage of the superoxide anion, O_2^- (Przedborski et al. 1996) and of Ca^{2+} (Packer et al. 1996). This Ca^{2+} rise inside the cell may lead to the activation of Ca^{2+} -dependent enzymes, such as neuronal nitric oxide synthase (nNOS), leading to the production of nitric oxide (NO). It has also been shown that O_2^- reacts with NO^-, generating peroxynitrite (Packer et al 1996, for review see Schulz et al. 1997). The key role that Ca^{2+} and nNOS play in the toxicity of MPP+ was shown in a study that demonstrated that pretreatment with nitro-L-arginine (an nNOS inhibitor) or the NMDA channel blocker, MK-801 (which prevents Ca^{2+} efflux), blocked the increase of hydroxyl radicals (OH^-) induced by MPTP administration to mice (Smith and Bennett 1997). These studies on MPP+ supported the theory that a defect in complex I, as has been found in PD patients (Nicklas et al. 1985, Schapira et al. 1990), can cause cell death and is likely to be involved in the pathogenesis of PD.

3.2.3 Excitotoxicity

The SN receives extensive glutamatergic innervation from the STN (Alexander and Delong 1990). Lesions of the STN have been shown to increase the firing rate of glutamatergic neurons to the SN (for review see Delong 1990), leading to direct excitotoxic damage. A second mechanism proposed to be involved in excitotoxic damage in PD is that a defect in mitochondrial energy metabolism could secondarily lead to slow excitotoxic neuronal death by making SN dopaminergic neurons more vulnerable to endogenous glutamate. It has been shown that NMDA antagonists can block the neurodegeneration induced by MPP+ in dopaminergic neurons (for review see Beal 1992). These studies suggest that excitotoxicity is likely to play a role in the pathogenesis of PD, but whether it is the primary causative agent or a secondary process remains to be determined (for review see Olanow and Tatton 1999).

3.3 Existing treatments for PD

Current treatments for PD treat the symptoms of the disease. They do not affect the pathological process, which continues unabated while the treatments are being taken. Existing treatments can be defined into two broad classes; drug treatment and surgical treatment.

3.3.1 Drug treatment

The main therapeutic drug used to control the symptoms of PD is L-dopa, the precursor of dopamine. Unlike dopamine, L-dopa crosses the blood brain barrier and is converted to dopamine within dopaminergic nerve terminals in the striatum (Adams and Victor 1993). In the early stages of PD, L-dopa treatment results in substantial improvements in symptoms, with most patients displaying a reduction in the severity of their symptoms (Cedarbaum et al. 1990). It has been calculated that only approximately 1% of orally administered L-dopa actually crosses the blood brain barrier, with much of it being broken down by peripheral decarboxylases. Thus L-dopa is usually administered in conjunction with a peripheral decarboxylase inhibitor (Sinemet) (Cedarbaum et al. 1990). Compounds which cause the release of dopamine, such as amatidine, have also been tested (for review see Stocchi and Olanow 2003). Because dopaminergic nerve terminals continue to degenerate, dopamine agonists such as bromocryptine are now being used as an alternative to L-dopa (for review see for review see Stocchi and Olanow 2003). Anticholinergic drugs such as Benzhexol are also used in an attempt to control the increased striatal cholinergic output which results from dopaminergic neuronal degeneration (for review see Stocchi and Olanow 2003). MAO-B inhibitors such as deprenyl and anti-oxidants such as have also been considered for future use in PD as MAO-B plays a role in MPP+ toxicity, thus it may be involved in the contributing to the ongoing degeneration seen in PD (for review see Stocchi and Olanow 2003).

3.3.2 Surgical treatment

Surgical treatment for PD can be divided into ablative procedures (which involves the lesioning of particular brain nuclei), deep brain stimulation (use of electrical stimulation to mimic the effect of a lesion but without causing damage) and restorative procedures (which aim to replace the lost cells and address the pathological process, see below). As discussed above, l-dopa therapy has been the mainstay of treatment for PD, however this is associated with disabling side-effects in a large number of patients (for review see Lang and Lozano 1998). In patients displaying severe functional impairment with resting tremor that does not respond to conventional drug therapy, three ablative procedures can be performed, thalamotomy, pallidotomy and subthalamotomy (for review see Olanow 2002). In general, thalamotomy has been used to improve unilateral resting tremor of the hand and involves lesioning the ventrointermediate nucleus of the thalamus (for review see Kupsch and Earl 1999, Olanow 2002). It has been reported that thalamotomy results in improvements in resting tremor in up to 90% of cases, but due to adverse side effects and morbidity due to haemorrhaging at the lesion site, this procedure is rarely used in the present day (for review see Kupsch and Earl 1999, Olanow 2002). Pallidotomy is favoured over thalamotomy because of the reported superior results obtained by placing lesions in the posteroventral portion of the GPi (Laitinen et al. 1992). Lesions of the STN remove the hyperactive output of this nucleus and studies have been carried out using this technique (Gill and Heywood 1997). Deep brain stimulation uses an electrical current to mimic the effects of ablative procedures. Its advantage over these procedures is that it does not involve permanent brain damage to the brain region, it is a reversible procedure and the degree and frequency of stimulation can be adjusted (for review see Koller et al. 1999).

3.4 Experimental therapies proposed for PD

As mentioned above, existing treatments for PD provide symptomatic relief but do not stop the pathogenic progress or aim to replace the cells that are lost in PD. In recent years extensive work has focused on addressing the above issues. Proposed experimental therapies can be broadly classified into two groups;

1. Replacing the lost dopaminergic neurons by transplantation of a population of dopaminergic neurons.
2. Preventing or at least slowing the loss of dopaminergic neurons in the SN by the use of neurotrophic factors.

3.4.1 Neural transplantation

As previously discussed, current pharmacological and surgical therapies for PD aim to provide symptomatic relief for the patient and do not aim to reconstruct the damaged circuitry as a result of dopaminergic degeneration in the SN. Neural transplantation aims to do just this, by replacing the missing dopamine neurons to restore the missing cells and hopefully lead to circuit reconstruction. The idea that neural transplantation might be a suitable therapy for the treatment of PD emerged when it was shown that grafts of embryonic rat ventral mesencephalon (VM – the embryonic SN) placed in either the lateral ventricle (Perlow et al. 1979) or a cortical cavity of adult rats (Bjorklünd and Stenevi 1979) resulted in improvements in tests of motor function and that surviving dopaminergic neurons were present within the grafts. It has subsequently been shown that the age of donor tissue (Barker et al. 1999) and the site of implantation of the graft within the host striatum (Dunnett et al. 1983) is critical for a functional effect to occur. However although a functional improvement was seen in these initial grafting studies, it has subsequently emerged that the survival of the grafted neurons is low, with only 2-5% of the implanted cells surviving the procedure (for review see

Barker and Dunnett 1999). A variety of methods have been employed in an attempt to increase the numbers of surviving dopaminergic neurons in transplants in animal studies, including optimising the tissue preparation prior to grafting (Emgard et al. 2002), and treating the graft with a variety of agents designed to prevent cell death. These agents have included anti-oxidants such as the lazaroids (Nakao et al. 1994), caspase inhibitors (Schierle et al. 1999), calcium channel blockers (Kaminski-Schierle et al. 1999) and a variety of neurotrophic factors (for review see Brundin et al. 2000), one of the most notable being glial cell line-derived neurotrophic factor (GDNF), as discussed below (Lin et al. 1993, Sinclair et al. 1996).

3.4.2 Adrenal medullary grafts

The first source of tissue considered for clinical transplantation in PD was the adrenal medulla (AM), due to the relatively high cathecholamine content of this tissue, which also contains dopaminergic neurons. The first clinical trials that were undertaken showed that although the grafted AM tissue resulted in an initial improvement in motor function, this effect was not permanent and the benefit usually disappeared after 18 months (Cahill and Olanow 1990). The technique was also found to cause high morbidity due to the two surgical procedures which were necessary; an abdominal procedure to obtain the AM tissue, followed by a neurosurgical approach to graft the tissue to the patients brain (for review see Quinn 1990). This finding, along with postmortem data that showed minimal graft survival of the AM tissue (Quinn 1990), have lead to the technique being discontinued due to the minimal beneficial effect to the patient and the risk involved.

3.4.3 Embryonic ventral mesencephalic grafts

The success of early grafting experiments using embryonic VM in rats (Bjorklünd and Stenevi 1979; Perlow et al. 1979) suggested that grafts of embryonic human VM might be a suitable donor tissue to use for clinical transplantation in PD. In the first trials carried out, the patients showed little clinical benefit (Lindvall et al. 1989). However, following refinement of the surgical techniques, studies have shown that grafted embryonic VM can survive in the human PD brain and integrate into the host striatum neural network (Lindvall et al. 1994) as well as providing long lasting relief of symptoms (Kordower et al. 1998). However these did not include a placebo group, which was suggested to be ethically unacceptable, and this sparked a debate that still continues (for review see Dekkers and Boer 2001). Results from the first double-blind placebo-controlled trial of transplantation of embryonic VM in PD were published in 2001 and showed that younger patients (<60 years) improved in the years following the transplant while the older group (>60years) did not (Freed et al. 2001). This trial provided conclusive evidence that the beneficial effect of grafting embryonic VM to the human PD brain was mediated by the grafted tissue. However as shown by experimental studies (for review see Barker and Dunnett 1999), few of the grafted neurons survive the transplantation procedure. The result of this is that approximately 8-10 embryos are required to complete a bilateral transplant in a PD patient (Olanow et al. 1996) making it logistically very difficult. Also the use of embryonic foetal tissue has raised ethical issues (Ferguson 1994, for review see Dekkers and Boer 2001). As a result of these practical and moral difficulties, the widespread adoption of clinical neurotransplantation for PD is unlikely unless alternative sources of donor tissues are found.

3.4.3 Neurotrophic factor application

A wide number of neurotrophic factors have been examined for potential use in PD. GDNF was initially purified from a rat glioma cell-line supernatant (Lin et al. 1993) and is a distant member of the transforming growth factor (TGF)-β superfamily (Krieglstein et al. 2002). GDNF plays numerous roles during development outside the nervous system. It acts as a morphogen in kidney development and regulates the differentiation of spermatogonia (for review see Airaksinen et al. 2002). GDNF has also been shown to be widely expressed in the adult brain, including the striatum and SN (Springer et al. 1994, Kawamoto et al. 2000). Although GDNF mRNA has not been detected in the developing rat VM at E14 (Krieglstein et al. 1995a), GDNF has been shown to be crucial for the survival of post-natal midbrain dopaminergic neurons (Granholm et al. 2000). Also, numerous studies since the initial characterisation of GDNF have shown it to be a potent dopaminergic neurotrophin *in vitro* for E14 VM dopaminergic neurons (Lin et al. 1993, Krieglstein et al. 1995a, 1995b). GDNF also improves the morphological development of these neurons (Widmer et al. 2000). Subsequently it was shown that intrastriatal administration of GDNF in a rat model of PD partially protected the degenerating dopaminergic neurons and spared their terminals in the striatum (Sullivan et al. 1997, 1999, Hurley et al. 2004). In non-human primates, GDNF administration to the striatum was shown to improve motor disabilities following MPTP administration (Costa et al. 2001). As such, GDNF has been proposed as a potential therapeutic agent for use in the treatment of PD. Initial results from a human trial showed GDNF to have no beneficial effect (Kordower et al., 1999). However more recent studies have shown a clear improvement in motor functions of PD patients after continuous intrastriatal infusions of GDNF (Gill et al. 2003). Use of GDNF as an adjunct to neural grafting, has shown that GDNF improves graft survival in animal

models of PD (Rosenblad et al. 1996, Sinclair et al. 1996, Wang et al. 1996, Sullivan et al. 1998).

3.5 Alternative cell sources for PD

Alternative sources of cells that have been considered for use in PD can be divided into those cells that are genetically engineered to provide molecular replacement of lost dopamine and those that aim to replace the lost dopaminergic neurons.

3.5.1 Embryonic neural stem/progenitor cells (NPCs)

In the developing and adult brain, neural stem cells exist which give rise to progenitor cells which are capable of differentiating into the major cell types of the nervous system- neurons, astrocytes and oligodendrocytes (for review see Gage 2000; see Figure 3.1). These neural stem cells have been isolated from the embryonic mouse (Reyonlds et al. 1992), rat (Svendsen et al. 1995) and human (Carpenter et al. 1999). CNS stem cells can proliferate in culture in response to epidermal growth factor (EGF) and basic fibroblastic growth factor-2 (FGF-2), generating structures called "neurospheres", which are free-floating spherical aggregates. Cells in these neurospheres express the intermediate filament protein nestin, which is used as a marker of multipotential stem cells (Frederiksen *et al.* 1988). Cells derived from these neurospheres can subsequently give rise to neurons, astrocytes and oligodendrocytes (for review see Gage 2000). Thus these cells can be used to study lineage specification from neural stem cells and have the potential to generate cells for transplantation approaches to the treatment of neurodegenerative disease, including PD (for reviews see Rossi and Cattaneo 2002, Svendsen and Smith 1999).

NPCs from the developing mouse (Studer et al. 1998), rodent (Ostenfeld et al. 2002) and human (Carpenter et al. 1999, Ostenfeld et al. 2002) midbrain have been

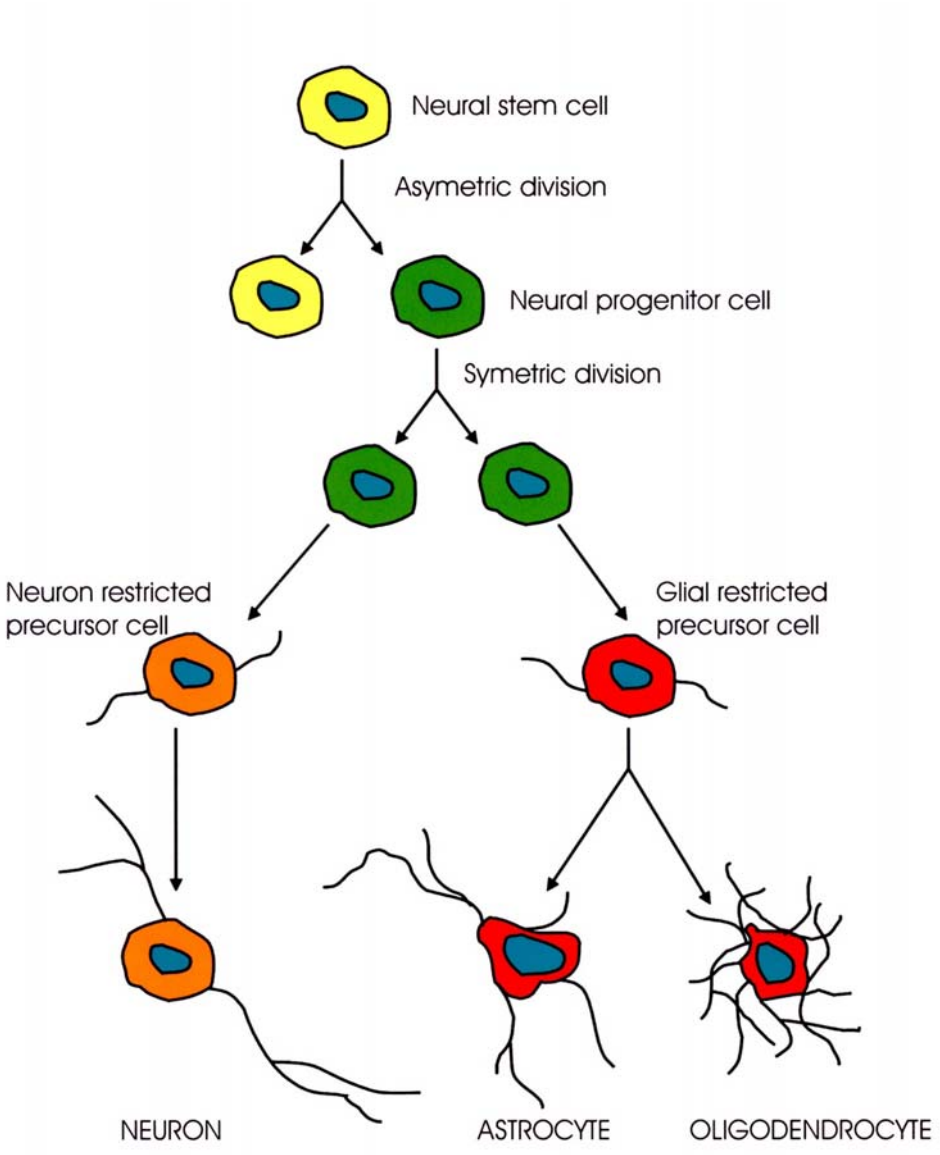


Figure 3.1:

Schematic representation showing the lineage tree of a neural stem cells as it self-reviews to give rise to itself and lineage restricted progeny. These lineage restricted progeny then differentiate to generate the major cell types of the nervous system, that is neurons, astrocytes and oligodendrocytes.

shown to have the potential to differentiate into tyrosine hydroxylase (TH; the rate-limiting enzyme involved in the synthesis of dopamine)-expressing dopaminergic neurons *in vitro*. As such, it was proposed that transplantation of these cells to the PD brain would result in functioning grafts containing dopaminergic neurons. Expanded human NPCs were shown to differentiate into mature neuronal and glial phenotypes when transplanted to the striatum of 6-hydroxydopamine (6-OHDA) lesioned rats, with a small number of dopaminergic neurons also being generated (Svendsen et al. 1997). Subsequently a number of groups have tried to enhance dopaminergic differentiation from NPCs. Riaz et al. have shown that expanded human NPCs from VM of 7-8 week old foetuses generate approximately 12% dopaminergic neurons when induced to differentiate (Riaz et al. 2002). It was shown that treatment of the cells during their differentiation with a combination of brain derived neurotrophic factor (BDNF; 10ng/ml), dopamine (10μM) and forskolin (10μM) increased the numbers of cells expressing TH to approximately 60% of total cells (Riaz et al. 2002). It has also been shown that BDNF, dopamine, forskolin and GDNF can induce the expression of TH in rat cortical NPCs (Theofilopoulos et al. 2001). Ling et al. showed that efficient generation of dopaminergic neurons from expanded mesencephalic rat NPCs could be achieved by treating the differentiating cells with interleukin (IL)-1, IL-11, leukima inhibitory factor (LIF) and GDNF. Addition of this cytokine mixture, along with mesencephalic membrane fragments and conditioned medium from striatal cultures, resulted in 20-25% of the overall population being immunopositive for TH (Ling et al 1998). Similar cytokine mixtures have also been used to induce TH expression in expanded human mesencephalic NPCs, although in this study TH neurons represented only 1% of the total cell population (Storch et al. 2001). Co-treatment of mouse embryonic striatal NPCs with FGF-2 and conditioned medium from the B49 glial cell line resulted in approximately 20% of the neurons expressing TH (Daadi and Weiss

1999). It has also been shown that dopaminergic neurons generated from expanded rat NPCs exhibit the morphological and functional characteristics of embryonic VM dopaminergic neurons (Storch et al. 2003).

Although previous work reporting the grafting of expanded undifferentiated NPCs to the striatum has shown that dopaminergic neurons can be generated in vivo (Svendsen et al. 1997), differentiation of the NPCs in vitro prior to grafting may be a more logical approach to generate large numbers of dopaminergic neurons. One report documenting such an approach was published in 1998 by Studer et al., who expanded E12 NPCs from the rat VM for 8 DIV with 10ng/ml FGF-2 and 1% N2 before differentiating the cells prior to transplantation (Studer et al. 1998). Removal of bFGF-2 and differentiation for 7 DIV resulted in 18.7% of the total cell population expressing TH. Grafting of these pre-differentiated cells to the 6-OHDA lesioned striatum of rats resulted in a partial amerlioration of drug-induced rotation, a test of motor function (Studer et al. 1998). Wagner et al. showed that a Nurr1-overexpressing a neural stem cell line (c17.2) gave rise to significant amounts of dopaminergic neurons *in vitro* (Wagner et al. 1999). Nurr1 is an orphan nuclear receptor essential for the development of midbrain dopaminergic neurons (Zetterstrom et al. 1997, Saucedo-Cardenas et al. 1998). Nurr1 overexpressing C17.2 cells co-cultured with type 1 astrocytes from the ventral midbrain resulted in 40-60% of the cells expressing TH (Wagner et al. 1999). E12 rat VM neural stem cells differentiated in low oxygen (3%) also gave rise to significantly higher numbers of dopaminergic neurons than cells differentiated in atmospheric oxygen (20%) (Studer et al. 2000). Expanded mouse mesencephalic NPCs have also been shown to differentiate into dopaminergic neurons and to mediate behavioural recovery when transplanted to a rat model of PD (Sawamoto et al. 2001). These studies have shown that dopaminergic neurons generated from expanded NPCs could provide a realistic alternative source of tissue for transplantation in PD.

3.5.2 Embryonic stem (ES)- cells

ES cells are pluripotent cells derived from the inner cell mass of the embryonic blastocyst. They can be propagated *in vitro* and can differentiate into representative derivatives of all three embryonic germ layers both *in vitro* and *in vivo* (for review see Conley et al. 2004). Since the demonstration that ES cells could be differentiated into neurons and glia (Fraichard et al. 1995, Schuldiner et al. 2001), these cells have been proposed as an alternative source of cells to generate dopaminergic neurons for transplantation approaches to PD (Bjorklund et al. 2002). Subsequently it has been shown that ES cells can generate functional dopaminergic neurons *in vitro* (Lee et al. 2000). In this study, 71% of the total cell population were neurons, with 7% of these neurons being TH-immunopositive. Treatment of the differentiating ES cells with sonic hedgehog (Shh), FGF-2-8 and ascorbic acid (AA) increased the percentage of TH-positive neurons to 34% (Lee et al. 2000). Efficient generation of hindbrain serotonergic neurons was also reported in this study (Lee et al. 2000). Efficient generation of dopaminergic neurons has also been reported by culturing ES cells on a layer of stromal cells (PA6), which result in 16% of the total cell population expressing TH (Kawasaki et al. 2000). It has also been shown that when undifferentiated ES cells are transplanted to the 6-OHDA-lesioned striatum in a rat model of PD, they develop into functional dopaminergic neurons and serotonergic neurons (Bjorklund et al. 2002). The ES cell-derived dopaminergic neurons reinnervate the host brain, reduce motor defects and restore physiological functional magnetic resonance imaging (MRI) response to dopamine-releasing agents (Bjorklund et al. 2002). However in 20% of cases recipient animals developed teratoma-like structures at the implantation site (Bjorklund et al. 2002). An ability to control the development of these teratomas is an essential pre-requisite before these cells can be used in a clinical setting. One possible

way to circumvent this problem would be to fully differentiate the cells *in vitro* before transplantation. Lee et al. used ES cells stably over-expressing Nurr-1 to generate differentiated dopaminergic neurons for transplantation to a rat model of PD (Kim et al. 2002). Approximately 78% of the total cell population were positive for TH when the Nurr-1 ES cells were treated with Shh and FGF-2-8. These differentiated cells were transplanted into the striatum of 6-OHDA lesioned rats, where they innervated the host brain, showed electrophysiological properties expected of neurons from the midbrain and induced significant improvements in tests of motor defects in an eight-week period following transplantation (Kim et al. 2002). Importantly, no teratomas were observed in this study, in contrast to that of Bjorklund et al. as described above. However, the long-term effects of these grafts must be established, in particular with respect to tumour formation.

3.5.3 Adult neural stem cells

One of the long-held beliefs was that the adult brain did not generate new neurons. However the demonstration that neurogenesis occurs in the adult rat hippocampus (Khun et al. 1996) and sub-ventricular zone (Luskin 1993), meant neural stem cells existed in the adult brain, which are capable of generating neurons and glia. It has been shown that cells isolated from the adult rat forebrain can be proliferated in culture and generate neurons and glia when induced to differentiate (Lois and Alvarez-Buylla 1993). NPCs can also be isolated from the adult rat SN, propagated *in vitro* in response to FGF-2 and generate neurons, astrocytes and oligodendrocytes *in vitro In vivo* these cells only generate glial cells in the SN (Lie et al. 2002). However when such NPCs from the SN were transplanted to the adult rat hippocampus they differentiated into neurons and glia (Lie et al. 2002). This suggests that appropriate neurogeneic signals do not exist in the

adult SN to induce these cells to follow a neuronal lineage. These findings, along with the demonstration that neurogenesis occurs in the adult human hippocampus (Eriksson et al 1998) and the fact that human NPCs have been isolated and propagated in vitro from the adult subventricular zone (Kukekov et al 1999), hippocampus (Kukekov et al 1999), cortex and amygdala (Arsenijevic et al 2001), lead to the proposal that adult human neural stem cells could be use in autologus transplantation in PD. This approach would circumvent the logistical, ethical and safety issues associated with transplantation of various other cell types, as mentioned above.

3.5.4 Retinal pigment epithelial (RPE) cells

The RPE forms part of the retina and consists of dopamine-producing support cells. It has been shown that transplantation of human RPE cells to the striatum in a 6-OHDA rat model of PD induces a minimal host immune response and improvements in motor deficits (Subramanian et al. 2002). Phase I clinical trials of transplantation of RPE in patients with PD has been carried out and the results are promising (for review see Watts et al. 2003).

3.5.5 Carotid body (CB) cells

The CBs contain neural crest-derived, highly dopaminergic glomus cells which function as arterial oxygen sensors, releasing dopamine in response to hypoxia (for review see Lopez-Barneo et al. 2003). These have been proposed as an alternative cell source for transplantation in PD, due to the possibility of because autologus transplantation. Transplantation of CB cells in the 6-OHDA rat model has proved that these grafts survive well and improve motor deficits (Espejo et al. 1998). The mechanism behind the functional recovery is unknown, however recent work has shown that the CBs contain high levels of GDNF, which may contribute to the trophic actions of CB

transplants on nigrostriatal dopaminergic neurons (Toledo-Aral et al. 2003). However information on the mechanism of action of these transplants is needed (for review see (Toledo-Aral et al. 2002).

3.5.6 Mesenchymal stem cells

These cells are generated from bone marrow and thus are also known as bone marrow stromal cells (BMSCs) (for review see Prockop 1997). These cells attracted much attention when it was shown that they could differentiate into neurons, astrocytes and oligodendrocytes *in vitro* (Sanchez-Ramos et al. 2000). When these cells were transplanted into rat brains, they engrafted and migrated into the host brain and developed many of the characteristics of astrocytes (Azizi et al. 1998; Kopen et al. 1999). As such it has been proposed that genetically engineered BMSCs could be used to deliver trophic factors such as GDNF. Human BMSCs transfected with the gene for TH were transplanted into a rodent model of PD and mediated significant functional recovery (Schwarz et al. 1999). The use of BMSCs as an alternative source of cells to deliver trophic factors is attractive because these cells could be used in autologus transplantation, since they could be isolated from the patient's own bone marrow.

3.6 The transforming growth factor-β superfamily

The TGF-β superfamily is a group of multifunctional cytokines that play diverse roles in many different tissues during development and in adulthood (for reviews see Sporn and Roberts 1990, Kingsley 1994, Herpin et al. 2004). The TGF-β superfamily is made up of a number of structurally-related molecules that are grouped into subfamilies based on sequence similarities (See Figures 3.2, 3.3, 3.4, 3.5). Some examples include the GDNF, TGFβ, activin (Act), and the Dpp-Vgr1-related (DVR) subfamilies (for reviews

see Krieglstein et al. 2002, Herpin et al. 2004). The DVR subgroup is further divided into the GDF-5 group, the bone morphogenetic (BMP)-2 group and the 60-A group, containing BMP-5, BMP-6, BMP-7 and BMP-8 (for reviews see Roberts and Sporn 1990, Kingsley 1994, Krieglstein et al. 2002, Herpin et al. 2004). Members of the TGF-β superfamily are synthesised as large precursor proteins that contain a hydrophobic N-terminal signal sequence, a prodomain of varying size and a C-terminal peptide. The precursor proteins are cleaved at a [RK]-X-X-[RK] site to release a mature C-terminal segment of 110-140 amino acids. The active signalling molecules are made up of hetero- or homodimers of this C-terminal region (for review see Kingsley 1994, Venkataraman et al. 1995, Herpin et al. 2004; see Figure 3.6A). All members of the TGF-β superfamily contain seven highly conserved cysteine residues in their C-terminal (Venkataraman et al. 1995), and all but one of the cysteine residues are involved in intramolecular disulphide bonds which result in a characteristic three-dimensional structure known as a cysteine knot motif (Venkataraman et al. 1995). The remaining cysteine residue is involved in intermolecular disulphide bond formation, allowing the formation of active dimers (Daopin et al. 1992). However some members, namely, GDF-3, GDF-9 (McPherron et al. 1993) and BMP-15 (see below) lack this cysteine residue and are thought to function as active monomers (see Figure 3.6B).

3.6.1 Transforming growth factor-β superfamily receptors

TGF-β superfamily members act through two different types of serine-threonine kinase receptors, which have been classified as type-I and type-II receptors, based on their structural and functional properties (for review see Massague 1996; see below). In the absence of ligand, these receptors exist as homodimers at the cell surface. On ligand binding, a functional tetrameric receptor complex is assembled which consists of two

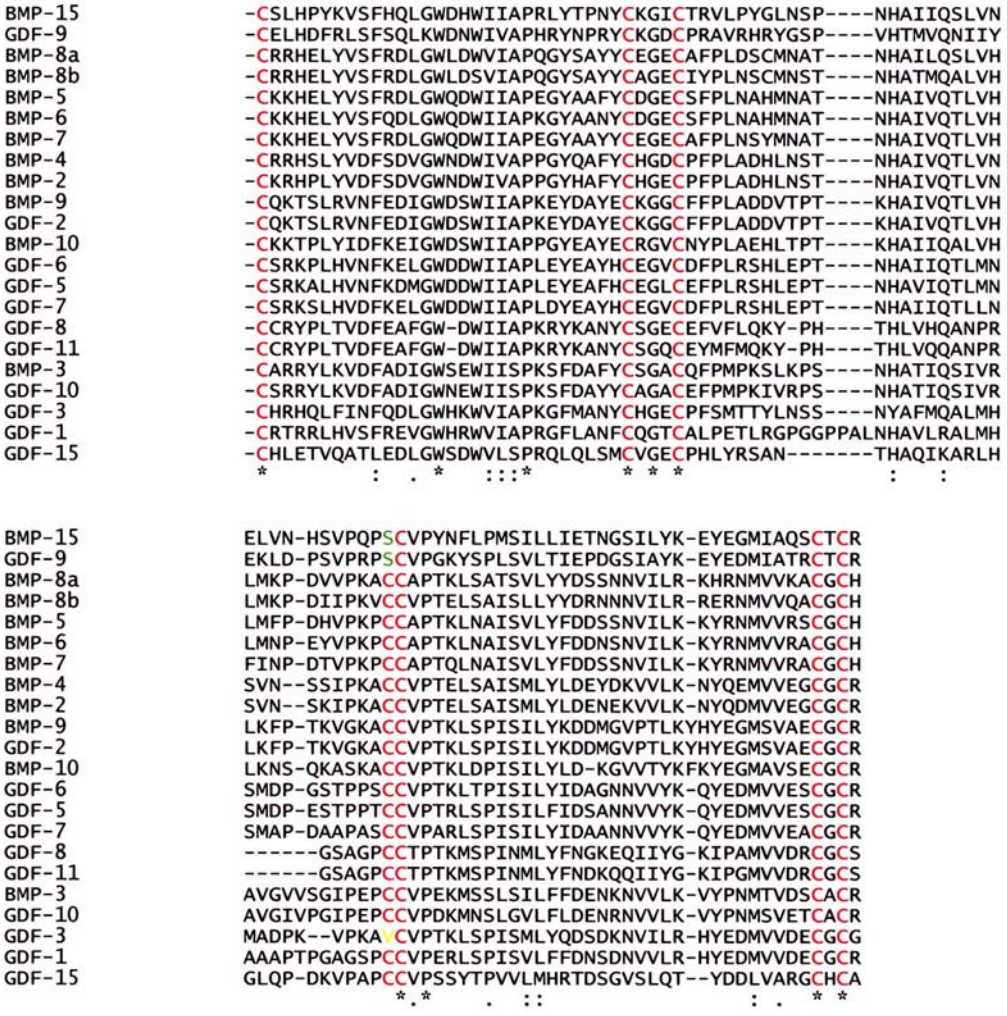


Figure 3.2: Alignment of known protein sequences of human BMP and GDFs beginning from the first conserved cysteine residue in the mature C-terminal protein. Alignment shows the classical seven conserved cysteine (C) residues that form the structure known as the "cysteine-knot motif". Protein sequences were obtained from www.ncbi.nih.gov and the alignment was performed using a web-based version of Clustal W™ from EMBL (http://www.ebi.ac.uk/clustalw/)

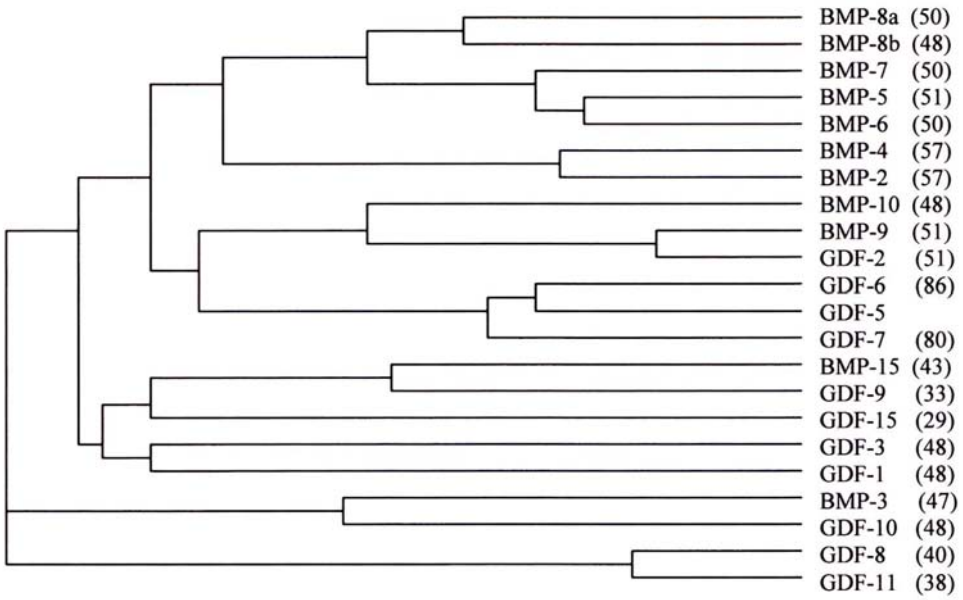


Figure 3.3: Phylogeny tree showing the evolutionary relationship between human BMPs and GDFs. Phylogeny tree was generated from data obtained from Clustal W™ alignment of the protein sequences shown in figure 3.2. Line length represents evolutionary distance. Numbers in brackets represent how similar the sequence in question is to the GDF-5 sequence (for example, "GDF10 (48)" means that the human GDF10 sequence is 48% similar to the human GDF-5 sequence). GDF6 and GDF7 share a high sequence similarity with GDF-% (86% and 80% respectively), showing that they belong to a subgroup within the DVR subfamily.

Figure 3.4: Cysteine residues are normally encoded by either TGT or TGC. This nucleotide sequence alignment of the cDNA encoding the C-terminal end of the GDF-5 protein shows the position of nucleotides encoding the cysteine residues. In general there is conservation of codon usage (either TGT or TGC) encoding for the conserved cysteine residues. However, differential codon usage is evident in the codon encoding the first conserved cysteine residue, with TGT present in the chicken sequence, while TGC is used in the human and mouse sequence. cDNA sequences were obtained from www.ncbi.nih.gov and the alignment was preformed using a web-based version of Clustal W™ from EMBL (http://www.ebi.ac.uk/clustalw/)

Figure 3.5: Alignment of known protein sequences of GDF-5 of various species beginning from the first conserved cysteine residue (C) in the mature C-terminal protein. The rabbit sequence was assumed to be 100% identical to the human sequence as previously reported (Sanyal et al. 2000). All sequences are identical expect for an amino acid substitution in the chick sequence from phenylalanine (F) to tyrosine (Y). Cysteine residues shown in green in the rat sequence are predicted. Protein sequences were obtained from www.ncbi.nih.gov and the alignment was performed using a web-based version of Clustal W™ from EMBL (http://www.ebi.ac.uk/clustalw/)

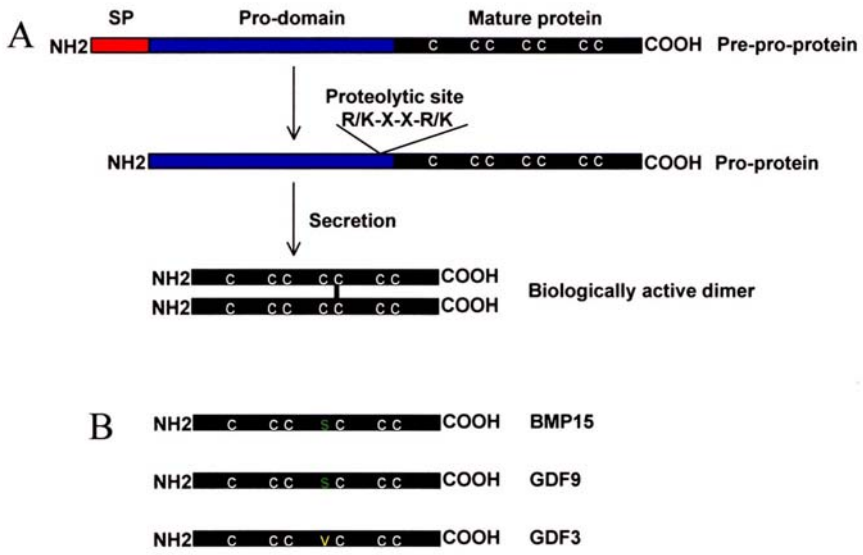


Figure 3.6: (A) Schematic showing the generation of a typical TGF-β ligand. Although most TGF-β ligands function as dimers, exceptions are GDF3 and GDF9 and BMP-15, which lack the cysteine residue required to form dimmers. (B) The mature protein of GDF3, GDF9 and BMP15, which are thought to function as active monomers. The cysteine residue (C) is replaced by a serine (S) in GDF9 and BMP15 and by a valine (V) in GDF3. *(SP=signal peptide)*

Part (A) of figure was adapted from (Herpin et al. 2004)

type-II receptors and two type-I receptors (for reviews see Massague 1996, Herpin et al. 2004). In mammals, only five type-II receptors and seven type-I receptors have been identified, whereas twenty nine ligands have been found (for review see Derynck and Zhang 2003). These receptors mediate a diverse array of signals from TGF-β superfamily members, by combining different type-I and type-II receptors in the tetrameric receptor complex to allow differential ligand binding or differential signalling in response to the same ligand (Derynck and Feng 1997). For example, interaction of Act receptor (ActR)-II with ActR-Ib mediates Act signalling, whereas interaction of ActR-II with BMP receptor (BMPR)-Ia or BMPR-Ib can mediate signalling by certain BMPs or GDFs (Nishitoh et al. 1996, Derynck and Zhang 2003). In the mouse, mutations in BMPR-Ia and BMPR-Ib as well as in ActR-Ib result in defects in gastrulation and mesoderm formation (Mishina et al. 1995, Gu et al. 1998, 1999). Type-II receptors are also required for gastrulation and mesoderm development, as demonstrated by mice with mutations in ActR-IIa and ActR-Ib (Song et al. 1999) or BMPR-II (Beppu et al. 2000), which fail to gastrulate or generate mesoderm. All three BMP receptors are expressed during brain development (Zhang et al. 1998, Panchision et al. 2001).

3.6.2 Signalling by transforming growth factor-β superfamily members

The BMPs, GDFs and Acts, along with the TGF-βs all act through a similar mechanism. Ligand binding to the type-II receptors causes these receptors to phosphorylate the cytoplasmic domains of the type-I receptors. Through a series of phosphorylations and protein interactions, these activate the smad effector proteins which move into the nucleus and affect transcription (for review see ten Dijke et al. 2000). Smads have been divided into three different categories based on their function in the cell: receptor

activated smads (R-smads), common mediator smad(s) (co-smad(s)) and inhibitory smads (I-smads) (for review see Roberts 1999). R-smads are numbered 1,2,3,5 and 8 and of these, smad 2 and 3 have been shown to mediate signals from the Act and TGF-β receptors, while smads 1,5 and 8 mediate signals from the BMP receptors (for review see Roberts 1999). Specificity of TGF-β and BMP signalling is determined by the "L3 loop" , a 17 amino acid region that protrudes from the core of the conserved MH-2 domain of the R-smads (Lo et al. 1998). The L3 loop is present in all R-smads, but differs in amino acid composition in smads 2 and 3 compared to smads 1, 5 and 8 (Lo et al. 1998). After phosphorylation of an R-smad by an activated type I receptor, the R-smads dissociate from the receptor and interact with smad 4 to form hetero-oligomeric complexes that undergo nuclear translocation, a critical event in the functioning of the smad proteins (for review see Miyazono et al. 1999). In the nucleus, smad proteins interact with DNA by directly binding to it and also indirectly by their associations with other DNA binding proteins (for review see Miyazono et al. 1999). The co-smad (smad 4) was first identified as a tumour supressor gene for pancreatic cancer that is either mutated or deleted in a significant percentage of pancreatic cancers as well as in a smaller number of colon cancers and breast cancers (Hahn et al. 1996). It has been demonstrated that smad4 specifically associates with each of the R-smads following their ligand dependant phosphorylation (Lagna et al. 1996). Inhibitory smads (Smads-6 and –7) lack the L3 loop and the C-terminal phosphorylation site of the R-smads and interact with both type 1 receptors and other smads (Roberts 1999). A rise in smad-6 and -7 mRNA occurs after TGF-β stimulation (Afrakhte et al. 1998) and it is thought that they act as in a negative feedback role, limiting the effects TGFβ superfamily members (Afrakhte et al. 1998, for review see Roberts 1999).

3.6.3 The bone morphogenetic proteins and the nervous system

As part of the DVR subgroup, BMPs represent the largest subfamily of the TGF-β superfamily (for review see Mehler et al. 1997). Initially identified due to their ability to induce bone, cartilage and bone marrow formation (Wozney et al. 1988), more recently these factors have been shown to play diverse roles in the development of the nervous system (for review see Mehler et al. 1997). One of the earliest events in which BMPs are involved is the regulation of neuralation during gastrulation. It has been shown that BMP-4 and BMP-7 are expressed by the ectoderm surrounding the dorsal blastopore lip and that they induce epidermal cell fate in the early embryo, while inhibiting neuralation (for reviews see Tanabe and Jessell 1996, Hemmati-Brivanlou and Melton 1997). Once gastrulation has been completed, BMPs act as key regulators in dorso-ventral patterning along the neuraxis (for review see Mehler et al. 1997). Various BMPs have been shown to be expressed along the dorsal midline of the neuraxis (Tanabe and Jessell 1996) and induce the expression of dorsal identity genes Pax1 and Msx1 in proliferating ventral precursor cells (Tanabe and Jessell 1996, Panchision et al. 2001). It is hypothesised that a dorso-ventral gradient is established along the neuroaxis, with BMPs expressed dorsally and various factors, the most important of which is Shh, which is secreted from the notochord, expressed ventrally (Ye et al. 1998, for review see Mehler et al. 1997). This gradient provides positional information for migrating cells (for review see Mehler et al. 1997).

BMPs have been shown to play diverse roles in determining cell fate during nervous system development (for review see Mehler et al. 1997). The cellular response to BMP treatment is diverse and ranges from apoptosis in early CNS precursors (Furuta et al. 1997, Mehler et al. 2000), neurogenesis or gliogenesis in mid-gestation CNS precursors depending on the time of addition of BMPs (Kalyani et al. 1998, Li et al.

1998, Mabie et al. 1999, Mehler et al. 2000), and glial differentiation in later embryonic or adult CNS precursors (Gross et al. 1996, Mabie et al. 1999, Mehler et al. 2000). In explant cultures of E13 mouse dorsal telencephalon, BMP-2, 4, 5, 7 treatment all caused mitotic arrest and increased the numbers of neurons, suggesting that they caused premature differentiation to a neuronal fate (Li et al. 1998). BMP-4 was also shown to increase neuritogenesis in these cultures; this required the presence of BMPR-Ia (Li et al. 1998). In agreement with this finding, BMP-2 has also been shown to promote neuronal differentiation from neural-restricted precursors isolated from the rat E13.5 spinal cord (Kalyani et al. 1998). In cultures of E16 rat proliferating cortical cells, BMP-2 caused cell cycle arrest and enhanced the numbers of neurons while decreasing the numbers of oligodendrocytes generated (Mabie et al. 1999). However, delayed addition of BMP-2 favoured the generation of astrocytes, while at the same time suppressing the generation of oligodendrocytes (Mabie et al. 1999), which suggests that, as precursors cells mature, the effect of BMP treatment can be different depending on the age of the cultured cells and on which precursor cells are present (Mehler et al. 2000). This fact is highlighted by studies showing enhanced generation of astrocytes as a result of BMP treatment. Proliferating O2A cells express the full complement of BMP receptors (Mabie et al. 1997). BMP-2, 4, or 7 treatment of these cells caused mitotic arrest and dramatically increased the numbers of astrocytes, while reducing the numbers of oligodendrocytes generated (Mabie et al. 1997). This effect was seen after a one h exposure to BMP-2, whereas if BMP-2 was added at a later stage, the cells were unresponsive (Mabie et al. 1997). This suggests that the role of BMPs is in the establishment of an astroglial phenotype and not in its maintenance. BMPs have been shown to act in synergy with LIF to induce differentiation of neuroepithelial precursor cells into astrocytes (Nakashima et al. 1999a). LIF is a member of the IL-6 family of cytokines which consists of IL-6, IL-11, ciliary neurotrophic factor (CNTF), oncostatin

M and cardiotrophin-1 (for review see Taga and Kishimoto 1997). Members of this family of cytokines signal through the membrane receptor gp130, which activates cytoplasmic Janus kinases, which lead to downstream activation of a transcription factor, STAT-3 (for review see Taga and Kishimoto 1997). BMP-2 and LIF co-treatment have been shown to stimulate a high transcriptional activation of the glial fibrillary acidic protein (GFAP) promoter (Nakashima et al. 1999a), agreeing with previous suggestions that the role of BMPs in astrogenesis is the induction of a phenotype rather than maintenance of the phenotype (Mabie et al. 1997). This synergism is mediated by a transcriptional activating complex consisting of STAT3 and Smad1, which is bridged by p300, a member of the cAMP responsive element-binding (CREB)-binding protein (CBP) family of transcriptional co-activators that have been previously shown to bind smad proteins (Nakashima et al. 1999b, Nishihara et al. 1998). Although BMP2 alone induces astrocytes from neuroepithelial precursors, LIF can accelerate this differentiation if the cells are cultured for longer time periods, (for example 3-7 days Gross et al. 1996 or 2 days Nakashima et al. 1999a). It has subsequently been found that gp130 activation is essential for BMP-2 induction of astrocytes from neuroepithelial precursors, as cells cultured from gp130 null mice, failed to generated any astrocytes in response to BMP-2 alone or in combination with LIF (Nakashima et al. 1999a). More recently, it has been shown that BMP-2 induces a switch in cell fate in proliferating telencephalic progenitors (Nakashima et al. 2001). BMP-2 was shown to induce the expression of the anti-neurogenic helix-loop-helix (HLH) transcription factors, Hes5, Id1 and Id3 by a smad-dependent mechanism (Nakashima et al. 2001). These findings have also been extended to BMP-4 and BMP-7, suggesting that BMP alteration of the fate of neuroepithelial precursors is via a common mechanism involving the induction of negative HLH transcription factors (Yanagisawa et al. 2001). However, the effects of BMPs on astrocyte induction may

also be regionally specific, as BMPs did not have this effect on neural precursors isolated from the spinal cord (Mekki-Dauriac et al. 2002), suggesting that neural precursors in divergent regions of the CNS respond differently to BMP treatment. Recently it has emerged that the effects of BMPs on the differentiation of CNS stem cells is also dependent on local cell density (Rajan et al. 2003). Neural stem cells isolated from the E14.5 rat cortex, predominantely differentiated into smooth muscle upon BMP-4 stimulation when the cell density is low, whereas glial differentiation predominated at higher local densities in response to BMP-4 (Rajan et al. 2003). Smad activation was shown to be necessary for smooth muscle differentiation, while STAT activation was necessary for glial differentiation (Rajan et al. 2003). In agreement with *in vitro* findings, transgenic BMP-4 over-expression during telencephalic development has been shown to increase the number of astrocytes, while reducing the number of oligodendrocytes generated (Gomes et al. 2003). BMPs have also been shown to be neurotrophic factors for developing sensory neurons from the dorsal root ganglion (Farkas et al. 1999), serotonergic neurons (Galter et al. 1999), midbrain dopaminergic neurons (Jordan et al. 1997; and see below) and striatal neurons (Gratacos et al. 2001, 2002). BMPs also regulate the postnatal differentiation of cerebellar cells, increasing neurite length and arborisation of developing granular cells (Angley et al. 2003).

3.6.4 The bone morphogenetic protein receptors and the nervous system

BMPRs have been reported not to be expressed at E9 in the developing mouse brain, but to be detectable by Western blot at E12. Their expression was shown to peak between E13 and P7, before decreasing with increasing gestational age (Zhang et al. 1998). Between E12 and E15 during mouse brain development, all three BMPRs are expressed in the ventricular zone (VZ) (Zhang et al. 1998), suggesting that BMP signalling may

play a role in neurogenesis at this stage of brain development, in agreement with previous findings (Kalyani et al. 1998, Li et al. 1998, Mabie et al. 1999, Mehler et al. 2000). By E16, BMPR-Ia is expressed in multiple structures of the mouse brain, including the striatal neuroepithelium, whereas expression of BMPR-Ib is limited to the frontal region of the neuroepithelium (Zhang et al. 1998). With increasing age, BMPR-Ia expression was found to increase in multiple brain structures whereas intensive BMPR-Ib expression was detected only in the anterior nuclei (Zhang et al. 1998). In the adult mouse brain, BMPR-Ia shows a more widespread expression pattern than BMPR-II and BMPR-Ib, with BMPR-Ib expression being limited to the anterior olfactory nucleus (Zhang et al. 1998). Mice overexpressing constitutively active (ca) BMPR-Ia at E11.5 and E14 showed expansion of the dorsal region of the neural tube along the entire rostral-caudal axis (Panchision et al. 2001). In contrast, mice overexpressing ca BMPR-Ib showed increased caspase-3 activation in the ventricular neuroepithelium between E9.5 and E12.5, showing that the principal early response to BMPR-Ib activation during brain development is apoptosis (Panchision et al. 2001), in agreement with previous *in vitro* studies (Furuta et al. 1997, Mehler et al. 2000). In later embryos (E13.5), the principal response to BMPR-Ib activation was differentiation to a neuronal phenotype (Panchision et al. 2001), also agreeing with previous *in vitro* studies (Kalyani et al. 1998, Li et al. 1998, Mabie et al. 1999, Mehler et al. 2000). Panchision et al. also showed that BMPR-Ia is expressed ubiquitously among neural precursors at all gestational ages, whereas BMPR-Ib is only expressed after E8.75 in the mouse, and is restricted to dorsal neural precursors (Panchision et al. 2001). In agreement with a role of BMPR-Ib in dorsalisation of neural precursors, Panchision et al. found that proliferating ventral neural precursors exposed to BMP-2 induced the expression of BMPR-Ib and Msx-1 (a dorsal identity gene) and that this effect was inhibited by Shh,

suggesting that BMPR-Ib activation induces a dorsalising phenotype in proliferating ventral neural precursors (Panchision et al. 2001; discussed in more detail in Chapter 9).

3.6.5 The growth/differentiation factors and the nervous system

The GDFs represent a subfamily of the TGF-β superfamily. Although the precise functions of many GDFs in the nervous system are unknown, recent data suggests that they play a role in the development of discrete neuronal populations.

GDF-1 is unusual among the TGF-β superfamily members, in that it is specifically expressed in the nervous system (Lee 1991). Transcripts for GDF-1 have been shown to be expressed in the developing mouse brain, and to increase during development, reaching maximal levels in the adult (Lee 1991). Within the adult mouse CNS, transcripts for GDF-1 have been shown to be expressed in the diverse regions, including the spinal cord, the cerebellum and the brain stem (Lee 1991). GDF-1 has also been shown to be strongly expressed in the hippocampus, cortex and thalamic nuclei during postnatal development in the mouse and rat brain (Soderstrom and Ebendal 1999), suggesting that it may play diverse roles during brain development, including a possible role in memory. GDF-1 transcripts have also been detected in the developing mouse VM (Wall et al. 2000), however an analysis GDF-1 null mice revealed no defect in VM dopaminergic neuron development in the VM of these mice (Mr. Kyle Sousa, personal communication). Activation of a smad2 dependant pathway by GDF-1 has been shown to control left-right patterning in the developing mouse embryo (Wall et al. 2000).

GDF-7 (BMP-12) was initially identified using degenerate primers for conserved carboxy-terminal domains of TGF-β superfamily members in mice (Storm et al. 1994). GDF-7 has been shown to be preferentially expressed in the primary motor area of the monkey neocortex (Watakabe et al. 2001). The role of GDF-7 in the motor cortex is

unknown. However, as the related factor, GDF-5, has been shown to be a dopaminergic neurotrophin (Krieglstein et al. 1995), it has been proposed that GDF-7 may be responsible for maintaining the dopaminergic innervation of the monkey neocortex (Watakabe et al. 2001) but no data exists to substantiate this claim. In cultures of E13 mouse dorsal telencephalon, GDF-7 and GDF-6 (BMP-13) have been shown to cause mitotic arrest of proliferating neuroepithelial precursors and to increase the numbers of neurons in these cultures (Li et al. 1998), suggesting that during development of the neocortex, GDF-7 may be involved in neurogenesis. A more defined role for GDF-7 was shown when it was demonstrated that this protein was expressed in the dorsal neuroepithelium at the mesencephalon/metencephalon boundary, the region that gives rise to granule neuron progenitors that migrate to the cerebellum (Alder et al. 1999). It was shown that GDF-7 could induce ventral cells of this region, that do not normally express markers of granule neuron progenitors, to switch on the expression of these genes and generate mature granule neurons when transplanted to the external granule layer of the postnatal mouse cerebellum (Alder et al. 1999). These findings have suggested that GDF-7 is involved in initiating the program of granule cell specification. It has also been shown that GDF-7 is essential for the development of a discrete class of commissural interneurons in the mouse spinal cord (Lee et al. 1998). Outside the nervous system, GDF7 has also been shown to be expressed in the uterus, kidney and spleen (Watakabe et al. 2001).

GDF-8 (myostatin) is mainly expressed in skeletal muscle, where it is thought to negatively regulate muscle growth (McPherron et al. 1997). Although GDF-8 expression is mainly restricted to skeletal elements in adult animals, it has been shown to be expressed in the developing zebra fish telencephalon (Radaelli et al. 2003).

Strengthening the argument that GDFs play a role in cerebellar development, GDF-10 has been shown to be almost exclusively expressed in the adult mouse

cerebellum (Zhao et al. 1999). GDF-10 was found to localise exclusively to the Purkinje cell layer of the cerebellum and was expressed in small cells surrounding the Purkinje cells (Zhao et al. 1999). However, GDF-10 knockout mice did not show any defect in cerebellar development and appeared to be grossly normal (Zhao et al. 1999). As such, the function of GDF-10 in the development of the cerebellum and embryogenesis as a whole may be redundant with that of other growth factors and remains to be determined. A subsequent report has shown that GDF-10 is also expressed in the postnatal mouse and rat cerebral cortex and hippocampus and that it eventually becomes restricted to the granule cell layer in the dentate gyrus from P21 onwards (Soderstrom and Ebendal 1999), suggesting that it may play a role in memory. Outside the CNS, GDF-10 is expressed in developing craniofacial structures and in the vertebral column (Zhao et al. 1999), and like GDF-7 (Watakabe et al. 2001), is also expressed in uterus, kidney and spleen (Zhao et al. 1999), suggesting that these two GDFs may share overlapping functions in some tissues.

Northern analysis has shown GDF-11 to be expressed only in dental pulp and in the brain in adult mice (Nakashima et al. 1999). During mouse embryogenesis on E16, GDF-11 was shown to be expressed in diverse regions of the developing brain including the thalamus, hippocampus, striatum and the Purkinje cell layer of the cerebellum (Nakashima et al. 1999). It is tempting to speculate that GDF11 in the striatum may function as a target-derived trophic factor for VM dopaminergic neurons, whose axons invade the striatum at E16 in the rat (Unsicker et al. 1996; see Figure 3.6). In the adult mouse brain, GDF-11 is expressed in the thalamus, the Purkinje layer of the cerebellum, the hippocampus and scattered cells in the midbrain and hindbrain (Nakashima et al. 1999). The expression of GDF-10 (Zhao et al. 1999) and GDF-11 (Nakashima et al. 1999) in the Purkinje cell layer of the cerebellum suggests that they may share overlapping functions and this may be a possible explanation for why GDF-10 knockout

mice have normal cerebellar development and function (Zhao et al. 1999). It has been shown that GDF-11 knockout mice all die within twenty four hs after birth and have a range of renal and palate anomalies (McPherron et al. 1999), although the exact cause of death remains unknown. GDF-11 knockout mice also displayed skeletal abnormalities, the reason being that GDF-11 is essential for specifying positional identity along the anterior/posterior axis (McPherron et al. 1999). GDF-11 is also implicated in regulating positional identity of spinal motor neurons (Liu et al. 2001) and in inducing reversible cell cycle arrest in neuronal progenitors in the olafactory bulb, thus negatively regulating the generation of new neurons (Wu et al. 2003).

GDF-15 (also known as macrophage inhibiting cytokine-1 (MIC-1)) (Bootcov et al. 1997; Böttner et al. 1999a, 1999b) has been shown to be expressed in the developing rat brain during the peri-natal period in diverse regions including the pons, medulla oblongata, striatum, cortex and hippocampus and to be expressed in purified cultures of astrocytes (Strelau et al. 2000). The most prominent site of GDF-15 synthesis in the peri-natal rat brain is the choroid plexus (Strelau et al. 2000). GDF-15 has also been shown to be a trophic and neuroprotective factor for E14 rat VM dopaminergic neurons *in vitro* and *in vivo* (Strelau et al. 2000). Subsequently GDF-15 has been shown to be upregulated in neurons and macrophages/microglia in response to a cortical cryolesion (Schober et al. 2001), suggesting that it may act as an anti-inflammatory molecule following CNS lesions. GDF-15 has also been shown to prevent low potassium-induced death of cerebellar granule neurons in culture (Subramaniam et al. 2003).

GDF-16 has also been shown to be expressed in the hindbrain and epibranchial placodes during *Xenopus* development (Vokes and Krieg 2000), however its function in these areas remain unknown.

3.6.6 Growth differentiation factor-5

The gene for GDF-5 is located on chromosome 2 in the mouse (Storm et al. 1994) and chromosome 20 in the human (Thomas et al. 1996). Mutations in this gene have been shown to be responsible for *Brachypodism (bp*) in mice (Storm et al. 1994) and a recessive chondrodysplasia in humans (Thomas et al. 1996), both of which are characterised by skeletal abnormalities restricted to the limbs and limb joints, suggesting a role for GDF-5 in limb development. *In vitro* and *in vivo* studies have shown that GDF-5 plays roles in mesenchyme aggregation and chondrogenesis (Hötten et al. 1996; Francis-West et al. 1999; Nakamura et al. 1999), tendon and ligament formation (Wolfman et al. 1997; Aspenberg and Forslund 1999), tooth formation (Morotome et al. 1998), morphogenesis of joints (Storm and Kingsley 1996) and angiogenesis (Yamashita et al. 1997, for review, see Buxton et al. 2001). GDF-5 signals through binding to two serine / threonine kinase receptors, the type I receptor BMPR-IB and either of the type II receptors, BMPR-II or ActR-II (Nishitoh et al. 1996). However recently it has emerged that Ror2, a orphan tyrosine kinase receptor, can act as a receptor for GDF-5 (Dr. P. Tylanowski, personal communication; see below). A role for GDF-5 in the brain was suggested by the initial detection of its mRNA in the embryonic mouse brain (Storm et al. 1994). Further experiments showed its presence in the cortex, brainstem, cerebellum and midbrain of newborn (P0) rat, albeit at low levels (Krieglstein et al. 1995b). Recent studies have reported neurotrophic and neuroprotective actions of GDF-5. This protein has been shown to increase the survival of E14 VM dopaminergic neurons *in vitro* to about the same extent as TGF-β3 and GDNF, well established dopaminergic neurotrophins (Krieglstein et al. 1995b). Furthermore, GDF-5 can attenuate dopaminergic neuronal death in E14 VM cultures induced by the neurotoxin MPP^+ (Krieglstein et al. 1995b) or by free radical donors (Lingor et al. 1999). GDF-5 was found to induce a dramatic increase in numbers

astrocytes in these cultures (Krieglstein et al. 1995b), suggesting that its action may be indirect, possibly by stimulating the production of glial-derived growth factor(s) that may be involved in the neurotrophic response. Another study showed that GDF-5 increased the number of serotonergic neurons detected in cultures of E14 rat raphe nucleus by upregulating the expression of serotonergic markers (Galter et al. 1999), suggesting that it may exert distinct actions on different cell populations. GDF-5 has also been shown to be a neurotrophic factor for sensory neurons from the dorsal root ganglia *in vitro* (Farkas et al. 1997).

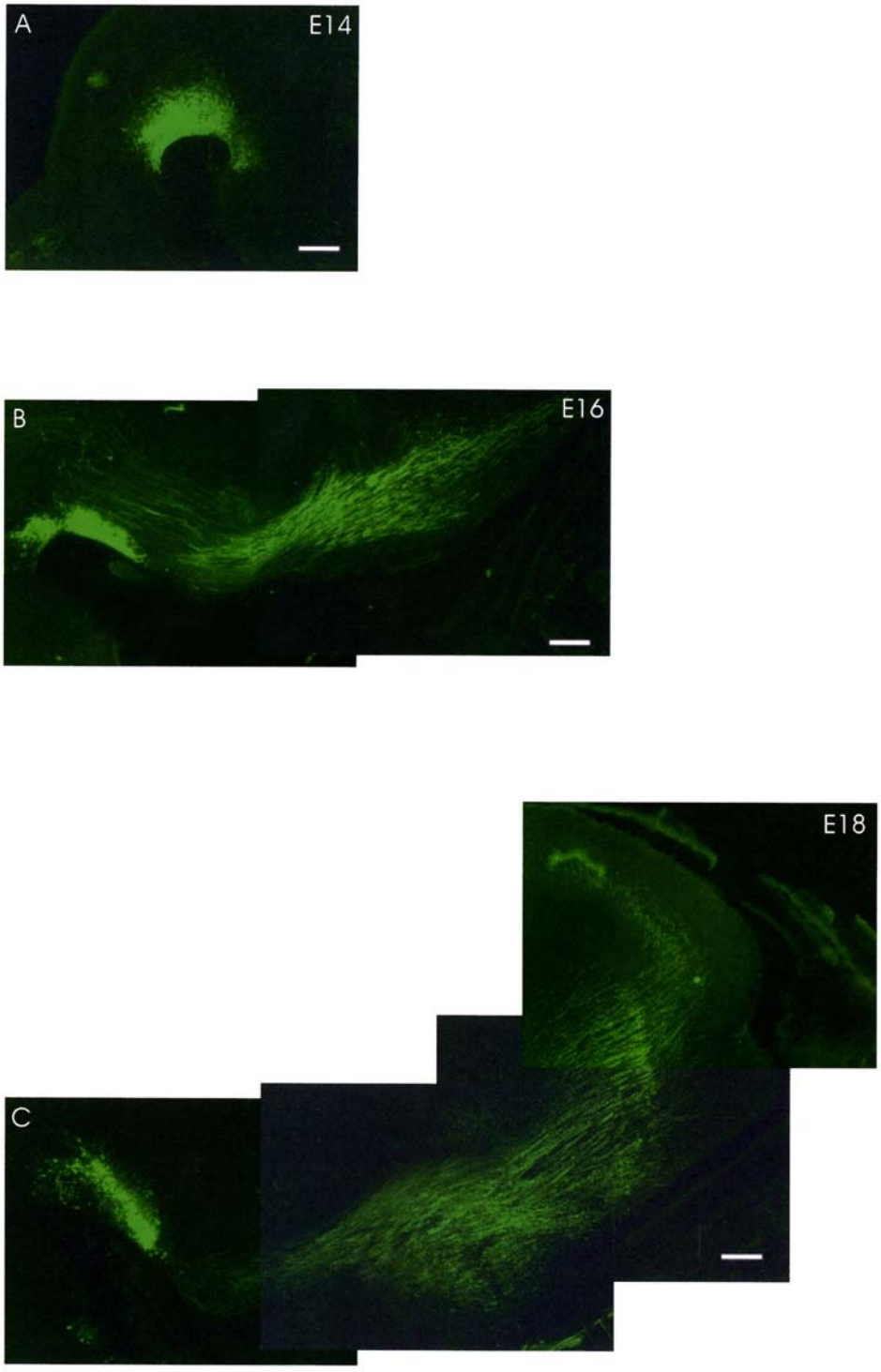
GDF-5 has also been shown to be a potent neurotrophic factor for dopaminergic neurons *in vivo*. Intrastriatal administration of recombinant human GDF-5 significantly attenuated damage induced by 6-OHDA-induced lesions of the adult rat nigrostriatal pathway, a model of Parkinson's disease (Sullivan et al. 1997, 1999; Hurley et al. 2004). Other studies showed that GDF-5 is at least as potent as GDNF in enhancing the survival and functional activity of foetal VM grafts in 6-OHDA-lesioned rats (Sullivan et al. 1998). More recently GDF-5 has been shown to be expressed in the developing rat brain with transcripts detected by *in situ* hybridisation in the Cajal-Retizus cells in the hippocampus (Kim et al. 2003). This suggests that GDF-5 may also play a role during hippocampal development although a precise role for GDF-5 during brain development remains to be determined.

3.7 The development of ventral midbrain dopaminergic neurons

In the rat VM dopaminergic neurons proliferate between E11 and E15 undergoing their final mitotic divisions on E14-15 (Hanaway et al. 1971; Lauder and Bloom 1974; Altman and Bayer 1981; Solberg et al. 1993; Bayer et al. 1995). In mice, the genesis of VM dopaminergic neurons follows a similar time course, with the neurons undergoing their final mitosis between E13 and E14 (Marti et al. 2002). In the rat, axons of

Figure 3.7:

Representative photomicrographs showing cryosections through the developing rat nigrostriatal pathway at (A) E14, (B) E16 and (C) E18, immunostained fro TH. Scale bar = 100μm


A
E14
B
E16
E18
C

nigrostriatal dopaminergic neurons start to invade the striatum at E16 and continue to grow pre- and post-natally (Unsicker et al. 1996, see Figure 3.6). This process of neurite outgrowth from the VM to the striatum has been shown to be controlled by spatially and temporally regulated chemoattractive (from the MFB and later the striatum) and chemorepulsive (from the brainstem and cortex) cues from the surrounding brain regions (Gates et al. 2004). However, the exact molecular nature of these cues remains to be determined. The development of VM dopaminergic neurons is regulated by a number of transcriptional regulators and secreted molecules that regulate the induction of a dopaminergic fate and subsequent maturation to a mature phenotype.

VM dopaminergic neurons are generated in the midbrain/hindbrain boundary under the influence of two major signalling centres, the isthmus and the floor plate (Ye et al. 1998). Among the secreted molecules known to regulate the development of VM dopaminergic neurons, two of the most important are Shh and FGF-2-8 (Ye et al. 1998). Shh, which is secreted from the floor plate, determines the position of VM dopaminergic neurons along the dorso-ventral axis, whereas FGF-2-8, which is secreted from the isthmus determines the position of these neurons along the antero-posterior axis (Ye et al. 1998). Gli proteins are zinc finger transcription factors that mediate the effects of Shh (Platt et al. 1997, Palma et al. 2004). The crucial role played by Shh in the development of VM dopaminergic has been demonstrated by mouse genetics studies. Overexpression of Gli-1 in the mouse leads to the development of ectopic dopaminergic neurons (Hynes et al. 1997), and in Gli-2 deficient mice, VM dopaminergic neurons fail to develop (Matise et al. 1998).

Members of the Wnt family of secreted glycoproteins are also expressed in the developing VM during the period of dopaminergic neurogenesis (Castelo-Branco et al. 2003). Wnt-1 has been shown to increase the proliferation of Nurr1-positive precursors, while Wnt5a increases the proportion of Nurr1-positive precursors that acquire a mature

dopaminergic phenotype and upregulates the expression of Pitx-3/Ptx-3 (see below) and c-ret (Castelo-Branco et al. 2003). Mice lacking Wnt-1 fail to develop a midbrain and anterior hindbrain (Danielian and McMahon 1996), the regions where engrailed (En)-1 and En-2 are expressed (Simon et al. 2001; see below) and it has been shown that Wnt1 signalling is necessary to maintain En expression (Danielanian and McMahon 1996). This finding is complemented by a study showing that mice lacking low-density lipoprotein receptor-related protein (LRP6), which forms a component of the Wnt signalling complex, also display midbrain abnormalities similar to those of mice lacking Wnt-1 (Pinson et al. 2000).

Another family of secreted molecules that are necessary to induce a VM dopaminergic cell fate are TGF-βs. Various TGF-β isoforms have been shown to expressed in the floor plate and notochord (Unsicker et al. 1996). Since Shh is also expressed in the floor plate (Ye et al. 1998), this suggests a role for TGF-βs in the specification of ventral identity within the developing neural tube. Recently, TGF-β2 and TGF-β3 as well as the TGF-β type II receptor, have all been shown to be expressed in the developing rat VM at E12.5 (Farkas et al. 2003). TGF-β3 has been shown to increase the numbers of cells expressing En-1, suggesting that TGF-β3 may play a role in the maintenance of a VM dopaminergic phenotype (Farkas et al. 2003). The induction of VM dopaminergic neurons by Shh was blocked by neutralising antibodies to TGF-β and vice versa, suggesting that these two factors require each other as co-factors to induce a VM dopaminergic cell fate (Farkas et al. 2003). As it has previously been shown that Gli3 can interact with smad proteins that mediate TGF-β signals (Liu et al. 1998), it is possible that cross talk between TGF-β and Shh signalling pathways may be involved in the acquisition of a VM dopaminergic cell fate. During the development of the neural tube in zebrafish, *cyclops* (a gene that encodes a protein related to the

TGF-β superfamily member, Nodal, has been shown to be necessary for Shh expression in the ventral aspect of the neural tube (Muller et al. 2000). This was dependant on smad2 (Muller et al. 2000), one of the smad proteins involved in mediating TGF-β signalling (Roberts 1999). The expression of BMP-2, -4 and –6 (Jordan et al. 1997) and GDF-5 (O'Keeffe et al. 2004a) in the developing rat VM suggests that these factors play a role in the development and/or subsequent maintenance of VM dopaminergic neurons. BMP-4 has been shown to induce En-1 expression in cultures from E12 rat VM; however, none of these cells expressed TH (Farkas et al. 2003), suggesting that additional co-factors are required to induce TH expression. BMP-4 and –7 expression in the wall of the dorsal aorta has been shown to be essential for the induction of TH in neural crest derived cells (Reissmann et al. 1996). However, the effect of BMPs on TH induction may be cell type-specific and temporally regulated, since other data has shown that BMPs are involved in the induction of cells with a dorsal identity in the hindbrain (Panchision et al. 2001; for review see Mehler et al. 1997).

Once the induction of a VM dopaminergic fate has been induced by extrinsic factors as described above, several regulatory genes are required for initiating and maintaining the expression of these dopaminergic neurons. Of the transcriptional regulators involved, Nurr-1 has been shown to be necessary for the development of dopaminergic neurons (Zetterstrom et al. 1997; Castillo et al. 1998). It has also been shown to directly activate the TH promoter (Sakurada et al. 1999). Over-expression of Nurr1 in ES cells has also been shown to upregulate a number of genes which are expressed by mature VM dopaminergic neurons, and these cells have been used to efficiently generate dopaminergic neurons (Kim et al 2002). Recent data has shown that Nurr1 induces a dopaminergic cell fate independent of a role in ventralization and genesis of neuroepithelial cells of the neural plate (Sonntag et al. 2004) and that other factors are necessary to induce a fully mature dopaminergic phenotype (Wagner et al.

1999). Mutations in the Nurr1 gene have been identified in familial PD, suggesting that Nurr1 is crucial to both the induction and maintenance of a mature dopaminergic phenotype (Le et al. 2003). Pitx-3/Ptx-3 is another homeodomain transcription factor that is exclusively expressed in VM dopaminergic neurons (Smidt et al. 1997) however the role of Pitx-3/Ptx-3 in the development of these neurons remains to be determined. Lmx-1b is a LIM homeodomain transcription factor that is initially expressed in the midbrain/hindbrain boundary and dorsal neural tube and later expressed in developing and adult VM dopaminergic neurons (Asbreuk et al. 2002). Lmx-1b has been show to induce the expression of Wnt-1 from the midbrain/hindbrain boundary (Adams et al. 2000) and Wnt 1 has been shown to increase the proliferation of Nurr1-positive precursors (Castelo-Branco et al. 2003), suggesting that the Nurr1-positive precursor pool is expanded (at least in part) through Lmx-1b mediated induction of Wnt1, prior to terminal differentiation. Lmx-1b knockout mice show normal development of Nurr1-positive, TH-positive VM dopaminergic neurons, but these cells lose the expression of Pitx3/Ptx3 (Smidt et al. 2000), suggesting that Lmx-1b acts upstream of Pitx-3/Ptx-3 and that the expression of these factors is independent of Nurr1 and TH expression in dopaminergic neurons. This conclusion is supported by the demonstration that Nurr1 knockout mice still express En and Pitx-3/Ptx-3 in the developing VM (Wallen et al. 1999). The engrailed homeodomain transcription factors, En-1 and En-2, are expressed in developing and adult VM dopaminergic neurons (Simon et al. 2001). In single and double knockouts of En1 and/or En-2 in mice, VM dopaminergic neurons are initially specified, but these quickly disappear, thus En-1 or En-2 is required for the maintenance of these cells (Simon et al. 2001). Recently the En-1and En-2 genes have been shown to be necessary to prevent apoptosis in midbrain dopaminergic neurons in mice, as dopaminergic neurons lacking these factors activated caspase-3 and underwent apoptosis (Alberi et al. 2004). En-1 and En-2 have also been shown to regulate the

expression of α-synuclein in mouse VM dopaminergic neurons (Simon et al. 2001). Since mutations in α-synuclein gene have been detected in PD (Polymeropoulos et al. 1997), it is possible that alterations in En regulation of α-synuclein production by En may be involved in PD, although this remains to be demonstrated. A recent study has also found that HNF3α, synaptotagmin 1 and Ebf3 genes are expressed during the development of mouse VM dopaminergic neurons (Thuret et al. 2004). HNF3α is a winged helix transcription factor (Duncan et al. 1998). It is expressed in the ventral midline from E9 onwards in the mouse in an overlapping expression pattern with Nurr1 and is expressed by VM dopaminergic cells before they become postmitotic (Thuret et al. 2004). In the adult mouse, HNF3α expression is restricted to dopaminergic neurons in the SN and VTA (Thuret et al. 2004), suggesting some regulatory function in adult VM dopaminergic neurons. Synaptotagmin I is a synaptic vesicle protein involved in the regulation of calcium-initiated exocytosis (Geppert et al. 1994). It is expressed in adult rat SN dopaminergic neurons (Glavan et al. 2000) and in human SN (Galvin et al. 2001). During the development of mouse VM dopaminergic neurons, synaptotagmin I expression begins at E14 and continues into adulthood (Thuret et al. 2004). The expression of synaptotagmin I by E14 VM dopaminergic neurons suggests that they are at least able to secrete dopamine in a calcium-dependant manner, and as dopamine has been shown to enhance dopaminergic differentiation from neural stem cells (Riaz et al. 2002), it is possible that synaptotagmin plays an indirect role in the acquisition of a fully mature VM dopaminergic neuronal phenotype. Ebf3 is a transcription factor containing a HLH dimerization domain and a zinc finger DNA binding domain (Garel et al. 1997). At E11 in the developing mouse VM, all TH-positive neurons have a Ebf3-positive nucleus (Thuret et al. 2004), however Ebf3 is only expressed from E11-E13 in these neurons (Thuret et al. 2004), suggesting that Ebf3 may be involved in induction of a VM dopaminergic phenotype and not in the maintenance of this phenotype. The exact roles

of these genes during the development of VM dopaminergic neurons remains to be determined.

3.8 The transforming growth factor-β superfamily and midbrain dopaminergic neurons

Following the demonstration that BMPs are expressed in the developing rat ventral mesencephalon at E13 and E15 (Jordan et al. 1997), these proteins have been shown to be neurotrophic factors for midbrain dopaminergic neurons (Jordan et al. 1997, Reiriz et al. 1999, Brederlau et al. 2002). Treatment of E14 rat VM cultures for 8 days in vitro (DIV) in serum-free medium with BMPs (with treatment beginning on 2DIV) resulted in increases in TH numbers. Differences in potency were seen between various BMP family members, with BMP-6 and BMP-12 being more effective than BMP-2, -4 and BMP-7 (Jordan et al. 1997). BMP treatment also stimulated proliferation of astrocytes in these cultures (Jordan et al. 1997). Inhibiting the BMP-induced increase in astrocytes was shown to abolish the surviving promoting effects of BMP-2 suggesting that BMPs may exert their neurotrophic indirectly through glia. A further study showed that addition of BMP-2 at the time of plating of E14 rat VM cultures led to an increase in dopaminergic cell numbers by 7 DIV (Reiriz et al. 1999). These findings show that early addition of BMPs to cultures of E14 VM results in improved survival. In the earlier study, no increase in astrocytes were observed after BMP treatment (Reiriz et al. 1999), unlike previous reports, which found that BMP treatment increased the numbers of astrocytes in these cultures (Jordan et al. 1997). Blocking of astrocyte proliferation and differentiation did not block the neurotrophic effect of BMP-2 in the Reiriz study, suggesting that BMP-2 exerts a direct effect on TH neurons. This is contrast to previous reports, which showed that prevention of the BMP-induced increase in the numbers of astrocytes abolished the increase in dopaminergic cell numbers (Galter et al. 1997). At

this time, it remains controversial about how the neurotrophic effects of BMPs are mediated (directly on neurons or indirectly via glia). However it has been shown that dopaminergic neurons in the adult rat substantia nigra express BMPR-II, suggesting that BMPs can exert a direct effect on dopaminergic neurons (discussed later). However, as astrocyte progenitors (O2A cells) also express a full complement of BMP receptors (Mabie et al. 1997), perhaps BMPs exert effects on neurons *and* astrocytes. Whether these effects are related remains to be determined.

BMP-2 has also been shown to enhance the morphological development of E14 VM dopaminergic neurons, leading to an approximately 1.4-fold increase in the number of primary neuritis and a five-fold increase in the total number of branch nodes per dopaminergic neurons, but no change in the somal area of these neurons (Reiriz et al. 1999). BMP-2 also improved the survival of E14 VM dopaminergic neurons in intrastriatal grafts in a 6-OHDA rat model of PD (Espejo et al. 1999). A further study examined the effects of BMP-3, -5, -6, -7 and GDF-5, -6 on E14 VM cultures (Brederlau et al. 2002). These factors were administered to the cultures after 6DIV and cultures were left to grow for a further 24 hs. Apart from BMP3, all of the factors examined induced an increase in the numbers of astrocytes and a depletion of oligodendrocytes in these cultures; however only BMP-5, BMP-6 and BMP-7 increased the numbers of dopaminergic neurons (Brederlau et al. 2002). These results are unlike those of a previous report, which showed that GDF-5 increased the numbers of dopaminergic neurons in E14 VM cultures (Krieglstein et al. 1995b). However in the earlier study, GDF-5 was added at an earlier time point (2DIV). TGF-β1-3, ActA and GDNF have also been shown to be neurotrophic factors for midbrain dopaminergic neurons and, unlike the BMPs, did not increase the numbers of astrocytes in E14 rat VM cultures (Lin et al. 1993; Poulsen et al. 1994; Krieglstein et al. 1995a). Other members of the GDNF subfamily have also been shown to be neurotrophic factors for E14 VM

dopaminergic neurons both *in vitro* and *in vivo*, with persephin (Milbrandt et al. 1998; Åkerud et al. 2002), neurturin (Horger et al. 1998) and artemin (Baloh et al. 1998) all having similar potencies to GDNF. GDNF has also been shown to influence the morphological development of E14 VM dopaminergic neurons (Widmer et al. 2000). GDNF treatment for 7 DIV resulted in a two-fold increase in total neurite length and a 1.5-fold increase in number of branch points per dopaminergic neuron (Widmer et al. 2000). Subsequently, treatment of E14VM cultures with GDNF and persphin for 5 DIV have been shown to result in a two-fold increase in neurite length per dopaminergic neuron (Åkerud et al. 2002).

3.9 Objectives of the present study

Although GDF-5 has been shown to be a dopaminergic neurotrophin no information regarding its expression during brain development or a detailed examination of its effects on dopaminergic neurons has been carried out. This study aims to address the following points;

- To characterise the expression of GDF-5 during rat brain development.
- To examine if GDF-5 is associated with the developing and adult nigrostriatal system.
- To examine the effects of GDF-5 on dopaminergic neurons *in vitro*.
- To examine if GDF-5 is involved in the induction of a dopaminergic phenotype.

4.0 MATERIALS AND METHODS

4.1 Tissue preparation for Western blotting

Protein extracts from the brains of rat embryos aged E11 to E20 were prepared as follows. Embryos were obtained by laparotomy from pregnant Sprague-Dawley rats, that had been lethally anaesthetised using sodium barbiturate (150 mg/kg, i.p.). The embryonic sacs were placed in a cold petri dish of Hanks' balanced salts solution (HBSS). Following removal from the yolk sac, the embryos were killed by decapitation. The brains of the embryos were dissected out using a dissecting microscope (Zeiss, Germany), and placed in ice-cold HBSS. The combined weight of all the brains was determined and then they were placed in a volume of homogenate buffer (see Appendix A) ten times the volume of their weight. Homogenisation was carried out using a hand held dounce homogeniser (AGB, Ireland). On completion of homogenisation, the samples were incubated on ice for 1 h and then spun for 12 min at 12,000 x g at 4ºC in a microcentrifuge (eppendorf), to remove insoluble debris. The supernatant was removed and stored at -20ºC until protein determination was carried out. P1 pups were killed by placing them on ice followed by decapitation and dissection of the brain. Rats ranging in age from P6 to adult were lethally anaesthetised using sodium barbiturate (150 mg/kg, i.p.). The brains and various tissues of adult rats were removed and homogenisation was carried out as described above for the preparation of the protein extract.

4.2 Preparation of culture extracts for Western blotting

Cultured cells were processed for Western blotting as follows. After the required period of time in culture, the medium was removed from the culture dishes and the cells were washed in cold HBSS. Following removal of the washing solution, an appropriate volume of homogenate buffer was added, as determined by the initial plating density

(50µl of homogenate buffer per 1 x 10^6 cells). The samples were transferred to a 2ml Eppendorf tube, incubated on ice for 1 h and then spun for 12 min at 12,000 x g at 4ºC in a microcentrifuge (Eppendorf), to remove insoluble debris. The supernatant was removed and stored at -20ºC until protein determination was carried out.

4.3 Determination of protein concentration in medium/cell/tissue extracts

The protein content in the samples was analysed according to the method of Bradford (1976) using 96-well flat bottom tissue culture plates (Nunc). Bovine serum albumin (BSA; Sigma, USA) standards ranging from 5-50µg/ml were diluted 1:2 with Bradford reagent (Bio-Rad) and the absorbance at 600 (A_{600}) was measured using a spectrophotometer. These readings were used to generate a linear standard curve. An aliquot of each protein sample of unknown concentration was diluted 1:100 with sterile distilled water. This solution was diluted 1:2 with Bradford reagent (Bio-Rad) and the absorbance at 600 (A_{600}) was determined. The protein concentration was determined by comparing the absorbance of each solution to the standard curve. Four protein determinations were carried out for each sample and the mean calculated.

4.4 Western blotting

Protein extracts were analysed by sodium dodecyl sulfate (SDS)-polyacrylamide gels, following the protocol of Laemmli (1970) with modifications. Extracts were analysed for the presence of GDF-5 by immunoblotting using a monoclonal GDF-5 antibody (P3/66). 30µg of protein from the extracts was diluted 1:2 in sample buffer (see appendix). The samples were boiled for 5 min prior to being loaded onto the gel. A 10% or 12% SDS-polyacrylamide separating minigel with a 5% stacking gel was used (Bio-Rad, Mini-PROTEAN® 3 Cell, for gel recipes see Appendix A). 50-100 ng recombinant human (rh)GDF-5 protein (Biopharm GmbH, Germany; in 10mM sodium

citrate buffer (see Appendix B) was used as a positive control. Biotinylated molecular weight markers (Amersham), diluted 1:10 according to the manufacturers instructions (see Appendix A), were loaded onto the gels to confirm molecular weights. The gel was assembled and run for 45-50 mins at 200V according to the manufacturers instructions (Bio-Rad, Mini-PROTEAN® 3 Cell, instruction manual) using the recommended running buffer (see appendix). Proteins were blotted to polyvinyl-idene difluoride (PVDF) membrane (Amersham). Electrophoretic transfer was carried out at a constant current of 100 volts for one h in transfer buffer (see Appendix A), according to the manufacturers instructions (Bio-Rad, Mini-PROTEAN® 3 Cell, instruction manual). After immunoblotting, efficiency of protein transfer was evaluated by staining the gels for residual protein bands with Coomassie Blue (see Appendix A). After transfer was completed, in some cases the PVDF membrane was stained with Ponceau S (see appendix) to confirm protein transfer. Following this, the membrane was blocked for 1 h in 5% w/v milk in 10mM phosphate buffered saline (PBS) (see Appendix A) with 1% tween-20 (PBST) (see AppendixA) at room temperature with gentle agitation on an orbital shaker. The blocking solution was removed and the membrane rinsed twice in PBST before being incubated at 4ºC overnight with the primary antibody (for a list of all antibodies and working dilutions, see Appendix A) in 1% BSA in PBST. The membranes were then washed (6 x 5 mins) in PBST and incubated at room temperature in anti-mouse peroxidase (for monoclonal antibodies; Amersham, 1:40,000), anti-rabbit peroxidase (for rabbit polyclonal antibodies; Amersham, 1:200,000) and anti-goat peroxidase (for goat polyclonal antibodies; Santa Cruz, 1:40,000) in 1% BSA in PBST for 1 h at room temperature with gentle agitation on an orbital shaker. Following washing as above, visualisation of the antibody complexes was carried out using ECL-Plus (Amersham) according to the manufacturers instructions. ECL-plus reagent was applied to the blot for 5 min, then drained from the membrane. The membrane was

sandwiched between two sheets of acetate and was placed in an autoradiography cassette and exposed to Hyperfilm^{TM} (Amersham). The film was developed and fixed manually (Kodak GBX developing and fixing solution, Sigma). After visualisation of the antibody complexes had been completed, the blot was stripped of all primary antibodies (western blot recycling kit, Chemicon, UK) and incubated in goat anti-β-actin (1:10,000, Santa-Cruz Biotech. USA) in 1% BSA in PBST for 1 h at room temperature. The blot was then washed as described above and incubated in anti-goat peroxidase (1:40,000, Santa-Cruz Biotech. USA) in 1% BSA in PBST for 1 h at room temperature. After washing as described above visualisation of the antibody complexes was carried out as above.

4.5 Cell culture

All *in vitro* work was carried out in a sterile laminar flow cabinet using strictly aseptic techniques.

4.5.1 Culture of GDF-5 producing CHO cells

The GDF-5 Chinese hamster ovary (CHO) cell line (MC-2), stably transfected with the human GDF-5 gene, was a kind gift from Biopharm GmbH, Heidelberg, Germany. This cell line was invented and subsequently patented [European patent: EP 0 866 125 A1] by Hoechst Marion Roussel, Ltd., Tokyo 107-8465, Japan. The MC-2 cell line was maintained in GDF-5-CHO cell line serum medium (see Appendix B). Cells were grown in sterile 25cm^2 flasks (Nunc®) at 37^0 C, 5% CO_2. Two thirds of the medium was discarded every 48 h and replaced with fresh medium (as above). Once the cells were confluent, the entire supernatant was discarded and the confluent cellular monolayer was either passaged or thereafter cultured in serum-free conditions in order to initiate expression of GDF-5 proteins.

4.5.2 Passaging of CHO cells

The medium was removed from the flask was then washed gently with 3ml of warm 10mM PBS. Then 4ml of warm 0.25% trypsin solution was added and incubated for 5 mins at 37^0 C. 0.5mg/ml of soyabean trypsin inhibitor was added and the flask was gently tapped to dislodge the cells from the base of the flask. 5ml of serum-enriched medium was added and cell suspension was centrifuged at 1000rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in 1 ml of serum-enriched medium. Estimates of total cell number were calculated using a haemocytometer (AGB) and 3 x 10^5 cells were seeded in a new, sterile 25cm^2 flask containing 10ml of warm serum-enriched medium.

4.5.3 Culture in serum-free medium for GDF-5 protein expression

When cells reached approximately 70% confluency, the serum-containing medium was removed and the cells washed gently with 3ml of warm 10mM PBS followed by washing with warm MEMα (Sigma). Then the confluent cellular monolayer was cultured in GDF-5-CHO cell line serum-free medium (see Appendix B). Two-thirds of this 'conditioned' medium was thereafter replaced every day, with fresh medium. Supernatant samples were labelled and stored at -20^0 C. The protein content in the medium samples was analysed according to the method of Bradford (Bradford, 1976) as described above and Western blotting using the P3/66 GDF-5 antibody was carried out as described above.

4.5.4 Preparation of cultures of E14 VM

E14 embryos were obtained by laparotomy from date-mated pregnant Sprague-Dawley rats (UCC Biological Services Unit) as described above. The brains were isolated from

the heads and the VM dissected as previously described (Dunnett and Björklund, 1992). The dissected pieces of VM tissue were incubated in 2mls of 0.1% trypsin solution for 15 mins at 37ºC. 0.5mg/ml of soyabean trypsin inhibitor was added and the tissue pieces were triturated using sterile flame-polished Pasteur pipettes for 10-15 strokes, with care being taken not to introduce air bubbles into the mixture. The cell suspension was then centrifuged for 5 mins at 1500 rpm. After discarding the supernatant, the tissue pellet was resuspended in 1ml of E14 plating medium (see Appendix B) using a P1000 pipette. 10µl of this cell suspension was diluted 1:100 in 1ml of fresh plating medium and an estimate of total cell number was calculated using a haemocytometer (AGB). The total number of cells in the suspension was calculated using the following formula;

$$\text{Cells/}\mu\text{l} = \left(^{\text{Number of cells counted in 10 fields}}/_{10}\right) \times \text{dilution factor} \times 10$$

Cells were plated onto poly-D-lysine (0.1mg/ml Sigma; see Appendix B) coated 13mm diameter coverslips (AGB) in 96 well plates (Nunc) and/or 3cm diameter four well culture dishes (Nunc) at 1 $x10^5$ cells/cm^2 and incubated at 37ºC with 5% CO_2. One half of the culture medium was changed every 2 days. For treatment of these cultures with GDF-5, GDF-5 was added directly to the cultures at a final concentration of either 1 or 10 ng/ml.

Fresh GDF-5 was added every 2 days when the medium was changed, at a final concentration of either 1 or 10 ng/ml. Cultures were grown for 2, 4 or 6 DIV, depending on the experiment being performed, before being fixed and processed for immunocytochemistry (see below).

4.5.5 Preparation of cultures of E12 VM

E12 cultures were prepared as described above, with the following exceptions. Due to the small amount of tissue, the dissociated cell suspension, after centrifugation at 1,500 rpm, was resuspended in 200µl of serum free E12 medium (see Appendix B). Estimation of total cell number was performed as described above. Cells were plated onto poly-D-lysine-coated (see Appendix B) 3cm diameter 4-well culture dishes (Nunc). 2 x 10^4 cells in 10µl were pipetted onto each well and the cells allowed to settle for 30 mins. 2ml of serum free E12 medium was added and the cultures incubated at 37ºC with 5% CO_2.

For TH induction experiment, growth factors were added directly to the cultures at a final concentration of 10 ng/ml. After 1 DIV, cultures were fixed and processed for immunocytochemistry (see below).

4.5.6 Brd-U labelling of cultures of E12 rat VM

To label proliferating cells in cultures of E12 VM, 10µM Brd-U (Sigma) was added directly to the culture medium for the final 5 h of the 24 h culture period. Cells were fixed and processed for immunocytochemistry (see below).

4.5.7 Preparation of neural progenitor cells from E12, E13 and E14 VM

All cell suspensions were prepared as described above. After centrifugation at 1,500 rpm, the cell pellet was resuspended in 200µl of expansion medium for neural progenitor cell culture (see Appendix B). Estimation of total cell number was performed as described above. Cells were seeded into 10cm^2 flasks (Nunc) at a density of 1 x 10^6 cells per flask in 5 ml of expansion medium. Fresh EGF and FGF-2 was added every day with half the medium being replaced on the fourth day of culture. For all experiments, cultures were grown for 6 DIV before being passaged for differentiation experiments.

4.5.8 Passaging of neural progenitor cells

After 6 DIV expansion, the cells rapidly proliferated into neurospheres. To passage the cells, the flasks were stood on their ends and the cells were removed using a 10ml pipette and transferred to a 20ml sterile tube. Attached cells were detached by scraping with a cell scraper and washing out the flask with expansion medium. After centrifugation at 1,500 rpm, the cell pellet was resuspended in 1ml of 0.1% trypsin solution for 15 mins at 37ºC. 0.5mg/ml of soyabean trypsin inhibitor was added and the mixture was triturated to a single cell suspension using sterile flame-polished Pasteur pipettes for 10-15 strokes, with care being taken not to introduce air bubbles into the mixture. The cell suspension was then centrifuged for 5 mins at 1500 rpm. After discarding the supernatant, the cell pellet was resuspended in 1ml of differentiation medium for neural progenitor cell culture (see Appendix B) and estimation of total cell number was performed as described above. Cells were plated onto poly-D-lysine-coated (see Appendix B) 3cm diameter 4-well culture dishes (Nunc). 1×10^5 cells in 20µl were pipetted onto each well and the cells allowed to settle for 30 mins. 2ml of differentiation medium (see Appendix B) was added and the cultures incubated at 37ºC with 5% CO_2. Cultures were grown for 6 DIV before being fixed and processed for immunocytochemistry.

4.6 Immunocytochemistry

4.6.1 Cultures

A similar immunocytochemical protocol was used for all cultures. The medium was removed and the cultures were washed in HBSS and fixed in 4% paraformaldehyde (Sigma) at room temperature for 20 mins. The paraformaldehyde was removed and the cultures were washed (3 x 10 mins) in 10mM PBS containing 0.02% Triton X-100

(PBS-Tx). For TUJ-1 staining, cultures were fixed in ice-cold methanol (Sigma) instead of paraformaldehyde, for 10 mins at room temperature. The methanol was removed and the cultures were washed (3 x 10 mins) in PBS-Tx. Following fixation, all cultures were incubated in blocking solution (5% normal horse serum, 0.2% Triton X-100, in 10mM PBS, pH 7.4; see Appendix B) for either 1 h at room temperature or overnight at 4ºC. Following this step, the blocking solution was removed and the cultures incubated overnight in the desired primary antibody (see Appendix B) at 4ºC. The primary antibody was removed, the cultures were washed (3 x 10 mins) in PBS-Tx and incubated at room temperature in anti-mouse FITC or anti-mouse TRITC (for monoclonal antibodies; Sigma, 1:50), anti-rabbit FITC or anti-rabbit TRITC (for rabbit polyclonal antibodies; Sigma, 1:50) or anti-goat FITC or anti-goat TRITC (for goat polyclonal antibodies; Sigma, 1:50) in blocking solution (see Appendix B) for 1 h at room temperature. Cultures were washed (3 x 10 mins) in PBS-Tx, counterstained with DAPI (10µg/ml; see Appendix B) for 4 mins and washed (3 x 10 mins) in PBS-Tx. Coverslips were mounted onto slides onto slides using mounting medium (Sigma; water:glycerol 1:1).

For double staining, cultures were processed as described above and incubated overnight in the desired primary antibody (see Appendix B) at 4ºC. Cultures were washed as above and incubated in the appropriate secondary antibody as above. Following washes, the cultures were re-incubated overnight in the second primary antibody in blocking solution (see Appendix B) at 4ºC. Following washing, the appropriate secondary antibody was applied and cultures were washed and processed as described above.

4.6.2 Embryonic brains

Sprague Dawley rat embryos, aged between E14 and E16 were obtained as described above. Embryonic pups were immersed fixed in 4% paraformaldehyde at 4°C for 24 hours followed by cryoprotection at 4°C for 24 hours in a 30% sucrose solution. 15µm cryosections were cut onto gelatin coated glass slides, or electrostatically charged superfrost glass slides (Nunc). These were washed three times in PBS immersed in blocking solution (Appendix B) for 1 h at room temperature. Sections were incubated in anti-rabbit-TH (Chemicon; 1:200) diluted in blocking solution at 4°C for 24 hours. Sections were then washed for (3 x 2 mins in 10mM PBS). Primary antibody binding was detected by appropriate anti-rabbit FITC (1:50: Sigma). Sections were again washed in PBS, and coverslipped using vectashield (vector).

4.7 Measurement of cellular morphology

For measurement of cellular morphology 50 neurons per treatment group (control, 1ng/ml and 10ng/ml GDF-5) from 3 independent experiments, were analysed using established stereological methods (Mayhew 1991). The two formulas used to calculate neurite length and cell somal area were as follows;

$$\text{Neurite length} = n \times T \times \pi/2$$

$$\text{Somal area} = n \times B$$

Where n = the number of times the neurites intersect the grid lines, T = the distance between the gridlines (taking the magnification into account), and B = the area associated with each point taking the magnification into account. An assessment of branching was done by counting the numbers of "nodes" per cell. Primary nodes were considered branches from the cell body, secondary nodes were considered branches of

primary neurites and so on for teritary and quaternary nodes. Data is presented as the average per dopaminergic neuron.

4.8 RNA isolation and RT-PCR

The VM from E12, E13 and E14 Sprague-Dawley rat embryos was dissected as described below. These tissue pieces were stored at 4ºC in RNA later solution (Sigma) until the RNA extraction was performed. For cultured cells, the medium was removed, the cultures washed in 10mM PBS and the cells were stored in RNA later solution as above. Total RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer's instructions. DNase (Promega) treatment of the extracted RNA was performed according to the manufactures instructions. 1µg of total RNA was reversed-transcribed using the RT ImProm II reverse transcription system (Promega). PCR reactions were carried out in a 25µl volume containing 50mM KCl, 1.5mM $MgCl_2$ (2mM for BMPR-Ia, 1mM for Ror2 and Msx1), 2µl cDNA, 10mM Tris pH 8.3, 200µM dNTPs, 800nM of each primer and 1U Taq. The following primers were used for BMPR-II (Chen et al. 2003) and BMPR-Ia (Panchision et al. 2000). All other primers were designed using Primer Select™ program.

Target mRNA	Sense Primer (5' to 3' direction)	Anti-sense primer (5' to 3' direction)
BMPR-II	GCTTCGCAGAATCAAGAACG	GTGGACTGAGTGGTGTTGTG
BMPR-Ib	ACACGCCCATCCCTCATCAGA	ACGCCACTTTCCCATCCACACT
BMPR-Ia	ACAGAAATTTATGGCACGCAA	CATACGCAAAGAACAGCATCTC
Ror2	ATCGCCCGCTCCAACCCTCTCATC	ATCCCCATCTTGCTGCCGTCTCG
Msx1	GGAGGCCGAGTTGGAGAAGTTGAA	AGAAGGGGTCGGAAGAGGGAGGAG
Nurr1	CTGTCTCCCGCCTTTCACTCTTCT	ATTTCGGCGGCGCTTATCCA
TH	TGTCACGTCCCCAAGGTTCAT	GGGCAGGCCGGGTCTCTAAGT

Thirty-four cycles of PCR were performed with the following temperatures; Denaturation 94°C for 30s, annealing at 57°C (BMPR-II and BMPR-Ib), 55.1°C (BMPR-Ia), 62.9°C (Ror2), 62.1°C (Msx1), 60.9°C (Nurr1) or 59.4°C (TH) for 30s and elongation at 72°C for 45s. A final elongation step was performed at 72°C for 5 mins. 20µl of PCR product was run at 100V for 45 mins on a 1% agarose-TBE gel.

4.9 Real time RT- PCR

GenBank rat cDNA sequences were used in PRIMER EXPRESS 1.0 (Applied Biosystems) for primer design. The following oligonucleotides were used (Quantum RNA classical 18S internal standard kit, Ambion, Austin, TX):

cDNA that had been FACS sorted into TH+ and TH- fractions from the VM of the E13.5 TH-GFP mouse (a kind gift from Ms. Nina Rawal, Karolinska Institute) was used in for realtime PCR analysis to examine BMP receptor expression. Real-time RT-PCR was performed in triplicate with 1 µ l of cDNA (1:10 dilution) and RT in a total volume of 25 µl. Each PCR consisted of 1X PCR buffer (Life Technologies) containing 3 mM $MgCl_2$ (Life Technologies), 0.2 mM deoxynucleoside triphosphates (Promega), 0.3 µ M each of the forward and reverse primers, 1 unit of Platinum *Taq* DNA polymerase (Life Technologies), and 1unit of SYBR green (Molecular Probes). The PCR was performed with the ABI Prism 5700 detection system (Applied Biosystems) and started with 94°C for 2 min, followed by 40 cycles, which each consisted of 94°C for 30 s, 60°C for 30 s , 72°C for 45 s, and 80°C for 15 s. A melting curve was obtained for each PCR product after each run to confirm that the SYBR green signal corresponded to a unique and specific amplicon.

Standard curves were generated in every 96-well plate for every real-time PCR run by using serial three-fold dilutions of a reverse transcribed RNA extract from an E11.5

whole mouse embryo. The resulting standard-curve plots were then used to convert the Cts (number of PCR cycles needed for a given template to be amplified to an established fluorescence threshold) into arbitrary quantities of initial template of a given sample.

The expression levels were obtained by subtracting the RT- value for each sample from the corresponding RT+ value and then dividing that number by the value of the housekeeping gene encoding 18S rRNA, obtained for every sample in parallel assays.

Each assay for a particular gene was repeated twice or three times in triplicate. Assays of 18S rRNA were run for each sample at the beginning and once or twice in the middle of assays to verify the integrity of the samples. The specificity of PCR primers was determined by BLAST run of the primer sequences. All PCR products were run in a 2% agarose gel to verify the size of the amplicon. The specificity of the PCR product was determined by sequencing the amplicon in random samples.

Statistical analysis of the results was performed by one-way ANOVA. A Fisher's protected least significant difference test was used post hoc to identify specific points at which the different developmental stages differed from the earliest stage only when significantly different interactions occurred. Significant difference for all tests was assumed at the level of $P < 0.05$ (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$).

5.0 EXPRESSION OF THE DOPAMINERGIC NEUROTROPHIN GROWTH DIFFERENTIATION FACTOR-5 IN THE DEVELOPING AND ADULT RAT BRAIN

5.1 Aims

- To characterise a monoclonal antibody to GDF-5 (P3/66)
- To examine the production of GDF-5 in a stably-transfected CHO cell line
- To examine the primary structure of the GDF-5 precursor protein and how it predicts processing of GDF-5.
- To examine the temporal expression of GDF-5 during embryonic and postnatal rat brain development.
- To examine GDF-5 expression in adult rat tissues, including different regions of the brain.
- To determine if GDF-5 is expressed in the developing rat nigrostriatal system.

5.2 Introduction

The TGF-β superfamily is a group of multifunctional cytokines that play roles in many aspects of cell growth, differentiation and development. It is made up of a number of structurally related molecules, including TGF-βs, Acts, inhibins, BMPs, GDFs and more distant members like GDNF (for reviews, see Sporn and Roberts 1990; Kingsley 1994; Herpin et al. 2004). The BMP family is further divided into the Dpp (BMP-2, -4, dpp-c, GDF-5) and 60A (60A, BMP-5, -6, -7, -8) subgroups, as well as members such as BMP-3, GDF-6 and Vg1, which do not belong to either of these subgroups (Krieglstein et al. 2002). The GDFs are thought to play diverse roles in many tissues (Caricasole et al. 1998; Settle et al. 2001; Vitt and Hsueh 2001; Witthuhn and Bernlohr 2001). In recent years, it has emerged that quite a number of these molecules, including GDF-1 (Lee 1991; Soderstrom et al. 1996; Wall et al. 2000), GDF-5 (Krieglstein et al. 1995b), GDF-7 (Watakabe et al. 2001), GDF-10 (Soderstrom and Ebendal 1999), GDF-11 (Nakashima et al. 1999), GDF-15 (Bottner et al. 1999a, 1999b; Strelau et al. 2000) and GDF-16 (Vokes and Krieg 2000), are expressed in the CNS. The functions of these growth factors in the CNS remain largely unknown, although recent studies have shown that GDF-5 and GDF-15 have neurotrophic actions (Farkas et al. 1997; Sullivan et al. 1997, 1999; Galter et al. 1999; Strelau et al. 2000; Subramaniam et al. 2003). GDF-7 may play a role in the development of the cerebellum (Alder et al. 1999) and GDF-11 has been shown to negatively regulate neurogenesis during olfactory bulb development (Wu et al. 2003).

The gene for GDF-5 is located on chromosome 2 in the mouse (Storm et al. 1994) and chromosome 20 in the human (Thomas et al. 1996). Mutations in this gene

have been shown to be responsible for *bp* in mice (Storm et al. 1994) and a recessive chondrodysplasia in humans (Thomas et al. 1996), both of which are characterised by skeletal abnormalities restricted to the limbs and limb joints, suggesting a role for GDF-5 in limb development. *In vitro* and *in vivo* studies have shown that GDF-5 plays roles in mesenchyme aggregation and chondrogenesis (Hötten et al. 1994; Francis-West et al. 1999; Nakamura et al. 1999), tendon and ligament formation (Wolfman et al. 1997; Aspenberg and Forslund 1999), tooth formation (Morotome et al. 1998), morphogenesis of joints (Storm and Kingsley 1996) and angiogenesis (Yamashita et al. 1997; for review, see Buxton et al. 2001). GDF-5 signals through binding to two serine / threonine kinase receptors, the type I receptor BMPR-IB and the type II receptor BMPR-II (Nishitoh et al. 1996).

A role for GDF-5 in the brain was suggested by the initial detection of GDF-5 mRNA in the embryonic mouse brain (Storm et al. 1994). Further experiments showed its presence in the cortex, brainstem, cerebellum and midbrain of newborn (P0) rat, albeit at low levels (Krieglstein et al. 1995b). Recent studies have reported neurotrophic and neuroprotective actions of GDF-5. This protein has been shown to increase the survival of embryonic day E14 VM dopaminergic neurons *in vitro* to about the same extent as TGF-β3 and GDNF, well established dopaminergic neurotrophins (Krieglstein et al. 1995a). Furthermore, GDF-5 can attenuate dopaminergic neuronal death induced by MPP^+ (Krieglstein et al. 1995b) or by free radical donors (Lingor et al. 1999). GDF-5 was found to induce a dramatic increase in the numbers of glial fibrillary acid protein (GFAP)-immunopositive cells in these cultures (Krieglstein et al. 1995b), suggesting that its neurotrophic action may be indirect, possibly by stimulating the production of glial-derived growth factor(s) which may be involved in the neurotrophic response. Another study showed that GDF-5 increased the number of serotonergic neurons detected in cultures of E14 rat raphe nucleus by upregulating the expression of

serotonergic markers (Galter et al. 1999), suggesting that it may exert distinct actions on different cell populations. GDF-5 has also been shown to be a potent neurotrophic factor for dopaminergic neurons *in vivo*. Intracerebral administration of recombinant human GDF-5 significantly attenuated damage induced by 6-OHDA-induced lesions of the adult rat nigrostriatal pathway (Sullivan et al. 1997, 1999; Hurley et al. 2004). Further studies showed that GDF-5 is at least as potent as GDNF in enhancing the survival and functional activity of foetal VM grafts in 6-OHDA-lesioned rats (Sullivan et al. 1998).

In this study, we examined the expression of GDF-5 protein in various organs of the adult rat, in particular the brain. Although neurotrophic actions of GDF-5 have been described, there is little information regarding the functions of GDF-5 in the brain. The presence of GDF-5 mRNA in the neonatal rat brain, including the midbrain, has been shown (Krieglstein et al. 1995b), however, very little is known about the expression of this protein in the developing or mature brain. The present study used Western blotting to characterise the expression of GDF-5 protein in extracts of developing and adult rat brain. Because of the reported neurotrophic actions of this protein on nigrostriatal dopaminergic neurons, the expression of GDF-5 in this region of the brain, both during its development and in the adult, was also investigated. Finally, GDF-5 expression in cultures of dopaminergic neurons from E14 rat VM was examined.

5.3 Results

5.3.1 Two different isoforms of GDF-5 precursor protein suggests active secretion

Western blotting using cell extracts and conditioned medium from the GDF-5-secreting CHO cells showed that GDF-5 exists intracellularly as a precursor protein, and not the mature protein of 15kDa (Figure 5.1A). Mature 15kDa protein was only detected in the conditioned medium (Figure 5.1B, lanes 3-7). This suggests that cleavage of the GDF-5 precursor protein to the mature form of GDF-5 may be a membrane-associated event. Primary structure analysis (www.expasy.org) of the GDF-5 precursor protein, revealed that amino acids 1-27 at the N-terminal were likely to be a signal peptide. When the molecular weight of the entire precursor protein was compared with that of the precursor protein minus the signal peptide, a 3kDa difference was observed between the two forms. The use of varying gel concentrations in western blotting experiments, allowed two distinct bands in the region of 55kDa to be discerned (Figure 5.4). When the molecular weights of these bands were calculated, they differed by 3 kDa, in agreement with the predicted molecular weights of the two isoforms of the precursor protein.

5.3.2 Active secretion of GDF-5 in a stably-transfected CHO cell line

The monoclonal antibody P3/66 was raised against the peptide fragment PLATRQGKRPSKNLKARC of the human GDF-5 protein. Alignment of this peptide fragment to the human GDF-5 protein sequence showed that it corresponds to amino acids 383-400, located near the C-terminal of the protein (Figure 5.2A). Western blotting using recombinant human GDF-5 protein (which migrates as a 25kDa dimer

under non-reducing conditions), showed that the antibody had a greater affinity for the reduced form of GDF-5, which migrates as a monomer of approximately 15kDa (Figure 5.2B). Thus all further western blots were carried out under reducing conditions. To further characterise this antibody, CHO cells stably transfected with the human gene GDF-5 gene (a kind gift from Dr. M. Hanke, Bioharm GmbH), were used. Western blotting using lysates from these cells showed that no 15kDa band was detected intracellular while a number of high molecular weight bands were detected (Figure 5.1A). Western blotting using conditioned medium from these cells showed that the cells secreted GDF-5 for at least 15 DIV (Figure 5.1B). As predicted, P3/66 antibody detected a band of 15kDa in conditioned medium from these cells (Figure 5.1B, lanes 3-8), while no band was detected in conditioned medium from mock transfected cells (Figure 5.1B, lanes 1-2). P3/66 antibody also detected a number of high molecular weight bands in the conditioned medium (see figure 5.1B).

5.3.3 Analysis of high molecular weight bands

The lowest of the high molecular weight isoforms detected intracellularly and in the conditioned medium from the GDF-5-expressing CHO cells was calculated to have a molecular weight of 54.6 kDa. Analysis of the molecular weight of the band found in brain samples found it to have a very similar molecular weight, i.e 55kDa. Prediction of the Mw of the human and mouse GDF-5 precursor proteins (www.expasy.org) showed that they have Mw of 55.4 kDa and 54.9 kDa respectively. This suggested that the band of approximately 55kDa which appeared in both conditioned medium from the CHO cells and in brain samples, corresponded to the monomeric form of the GDF-5 precursor protein. To confirm this, an immunoabsorption assay was carried out to test the specificity of binding of the monoclonal antibody P3/66 (Figure 5.3A, B). The antibody was incubated overnight in an excess of monomeric rhGDF-5 before being

used in a Western blot on extracts from adult rat midbrain and striatum. This resulted in the immunoreactive band for rhGDF-5 being extremely reduced in intensity (Figure 5.3B), indicating specific binding of the P3/66 antibody to GDF-5 protein. The immunoreactive bands for the midbrain and striatum tissue extracts were also greatly reduced in intensity, indicating that the band at ~55kD in the samples corresponded to the GDF-5 (precursor) protein (Figure 5.3A, B).

5.3.4 GDF-5 is expressed in the developing and adult rat brain

Western blots on samples prepared from various aged embryonic rat brains, showed expression of a distinct band of ~55kDa. Expression of the ~55kDa band was not present at E11, but appeared at E12 and increased in intensity up to E14, before decreasing gradually with increasing age, until only a very weak band could be detected at P1 (Figure 5.5A, B). Recombinant human GDF-5 was detected as a clear band at ~15kDa in each blot, corresponding to the processed monomeric (reduced) form of the protein (Figure 5.5A, B).

Postnatally, the expression of ~55kDa immunoreactive band increased in intensity with increasing age, reaching maximal levels in the adult brain. Whereas only a very faint band was present in whole brain extracts from P1 rat brain, bands of increasing intensity were detected in extracts from P6, P14 and P22 brain (Figure 5.5A, B). The band observed at P22 was of comparable intensity to that seen in extracts from adult rat striatum and whole brain (Figure 5.5A, B).

The changes in intensity of the GDF-5-immunoreactive bands in each blot were verified by normalising their intensity to that of the corresponding β-actin-immunoreactive band for each age.

5.3.5 Expression of GDF-5 is tissue-specific

Of the organs examined, GDF-5 was expressed in adult rat heart and brain, but not in lung, liver, kidney or spleen (Figure 5.6A, B). Expression in heart tissue was very intense. Within the adult rat brain, GDF-5 protein was detected in the cortex, striatum, midbrain and cerebellum (Figure 5.7A, B).

5.3.6 GDF-5 is expressed in the developing rat VM in vivo

Immunoreactive bands at molecular weight ~55kDa were also found in protein extracts from the developing rat VM. This expression was present at E12 and increased in intensity up to E14, at which time it was maximal (Figure 5.8A). Between E15 and P2, there was a gradual decrease in GDF-5 expression levels (Figure 5.8A). The time-course of expression of GDF-5 in the VM closely paralleled that of the expression of TH, a marker for dopaminergic neurons. TH expression became apparent at E13, peaked at E15 and was present at high levels at the later ages which were examined, that is E18, E19 and P2 (Figure 5.8B).

5.3.7 GDF-5 is expressed in the developing rat VM in vitro

GDF-5 protein was detected by immunocytochemisty in untreated E14 VM cultures at 2 DIV. Expression of this protein was widespread in many cells, which possessed the typical morphology of neurons. GDF-5 was found to be expressed throughout the cell bodies and processes (Figure 5.9A, B).

5.3.8 GDF-5 is expressed in the developing rat striatum in vivo

Immunoreactive bands at the molecular weight ~55kDa were found in protein extracts from the developing rat striatum. Strong expression of GDF-5 was observed in all ages examined (E14-E20), however expression decreased slightly with increasing age (Figure 5.10).

5.4 Discussion

5.4.1 Technical considerations regarding Western blotting for GDF-5

Members of the TGF-ß superfamily are synthesised as large precursor proteins that contain an N-terminal signal sequence and a prodomain of varying size. The precursor proteins are cleaved at a RXXR site to release a mature C-terminal segment of 110-140 amino acids. The active signalling molecules are made up of hetero- or homodimers of this C-terminal region (Kingsley 1994, Venkataraman et al. 1995). Mature GDF-5 is a dimer composed of two 12.5kDa monomers, which are linked by a disulphide bond (Hötten et al. 1994). In this study, rhGDF-5 consistently migrated at an apparent molecular weight of ~15kDa under reducing conditions, as has been reported previously (Hötten et al. 1996).

In the present study, a monoclonal antibody to GDF-5 detected a band of approximately 55kDa on Western blots of extracts from developing and adult rat brain and conditioned medium from GDF-5 expressing CHO cells, which matches the predicted molecular weight of the mouse precursor protein (Storm et al. 1994). An immunoabsorption assay confirmed the specificity of the monoclonal antibody P3/66 to GDF-5.

Mature GDF-5 was not detected in extracts from either developing or adult rat brain in this study. Since the antibody used is capable of recognising the mature form of GDF-5 (shown by Western blotting using the recombinant protein), it is probable that the levels of mature GDF-5 in the tissue lysates may be below the detection limit of this technique. Another member of the TGF-β superfamily, GDF-15, has recently been reported to be present in the mature form in brain tissue at levels which are too low to be detected by Western blotting (Strelau et al. 2000). This study detected an immunoreactive band at ~30 kDa, which corresponds to the GDF-15 precursor. These

results are supported by a previous study which showed that very low levels of mature GDF-15 exist intracellularly, while the precursor form is in abundance (Bootcov et al. 1997). Similarly, mature GDF-1 protein could not be detected using Western blotting on extracts of mouse embryonic tissue, while the precursor form was detected in abundance (Wall et al. 2000). Similar findings have also been found for BMP-4 transfected COS-1 cells (Constam and Robertson 1999). Our findings also show that intracellularly GDF-5 exists predominantly in the precursor form. The lack of mature GDF-5 intracellularly suggests that cleavage of the mature protein from the precursor protein may be a membrane-associated event, possibly mediated by subtilisin-like proprotein convertases, as has been shown for Nodal and BMP-4 (Constam and Robertson 1999). However this theory requires further investigation.

As discussed above, the immunoreactive band of ~55kDa which was found in brain tissue in the present study is likely to correspond to the GDF-5 precursor protein. However, given that no mature GDF-5 was detected in brain extracts, it is possible that no GDF-5 mature protein is made/secreted by cells in these tissues. An analysis of the mouse and human GDF-5 precursor proteins suggest the presence of a stretch of 27 amino acids at the N-terminal, which may function as a signal peptide. Cleavage of this signal peptide would result in two isoforms of the precursor proteins that would differ in molecular weight by 3.2 kDa. Our finding of two immunoreactive bands in the adult rat brain extracts, that differ in molecular weight by 3 kDa, suggests that cleavage of this signal peptide may occur *in vivo*. This suggests active cleavage of the GDF-5 precursor protein by intracellular proteases. It has been previously shown that the amino acid composition of the region of the pro-domain surrounding the R-X-X-R cleavage site determines the cleavage efficiency and that the pro-domain can influence the stability of the mature protein (Constam and Robertson 1999). Transfection studies have shown that, although the Nodal precursor is efficiently cleaved, no mature protein could be

detected in conditioned medium from these transfected cells, suggesting that mature Nodal is highly unstable. When the precursor domain of BMP-4 was fused to the mature part of nodal, mature Nodal protein could be detected in the conditioned medium from transfected cells (Constam and Robertson 1999). This suggests that the pro-domain can exert a stabilising influence on the mature protein. Our finding that mature GDF-5 was detectable in conditioned medium from transfected cells, and the presence of high molecular weight isoforms in both cell lysates and conditioned medium from transfected cells, suggests that the GDF-5 precursor domain may be involved in stabilising the mature GDF-5 protein.

5.4.2 GDF-5 expression in the adult rat heart

This study has found high levels of GDF-5 expression in the adult rat heart. A cardiac function for the BMPs has previously been suggested, since high levels of expression of BMP receptors have been found in the developing mouse heart (Böttner et al. 1999a) and various BMPs have been shown to play a critical role in cardiomyocyte differentiation (Monzen et al. 2002). Furthermore, ectopic expression of dominant negative BMPR-IA or BMPR-II receptors in *xenopus* embryos was found to reduce or prevent heart formation (Shi et al. 2000), supporting a critical role for the BMPs in cardiac development. Some members of the BMP family, such as BMP-10, are expressed specifically in the heart in the adult mouse (Neuhaus et al. 1999). Others, such as GDF-15, show a much wider expression pattern and are distributed in many adult tissues, including the heart (Böttner et al. 1999b). The present study has found that the detectable expression of GDF-5 in the adult rat is limited to the brain and heart. To our knowledge, this is the first demonstration of GDF-5 protein expression in the adult rat heart, suggesting a role for GDF-5 in cardiac function.

5.4.3 GDF-5 expression in the adult rat brain

The neuroprotective effects of intracerebral injection of rhGDF-5 prior to and following a 6-OHDA lesion of the adult rat nigrostriatal dopaminergic pathway *in vivo* have been reported (Sullivan et al. 1997, 1999; Hurley et al. 2004). The present study has found that GDF-5 is widely expressed in many regions of the adult rat brain, including the cortex, striatum, midbrain and cerebellum. The finding that GDF-5 is expressed in the adult rat striatum and midbrain, along with its documented neurotrophic effects on nigrostriatal dopaminergic neurons, suggests that it may play a role in the maintenance of the nigrostriatal system in the mature brain. One of the GDF-5 receptors, BMPR-II, has been shown to be expressed by dopaminergic neurons in the SN and VTA in the adult rat (Charytoniuk et al. 2000), supporting the idea that GDF-5 can exert a direct effect on dopaminergic neurons.

To our knowledge, the present study is the first report of GDF-5 protein expression in the adult rat cerebellum. The widespread expression of the BMP receptors (Soderstrom et al. 1996) and the expression of GDFs such as GDF-10 (Zhao et al. 1999) in the developing and adult mouse cerebellum suggest that the GDFs play a role in cerebellar function. The present study shows here that GDF-5 is expressed in the adult rat cortex, a region in which other GDFs, such as GDF-15 (Strelau et al. 2000; Schober et al. 2001) and GDF-7 (Watakabe et al. 2001), are expressed. Due to their widespread expression, the functions of GDF-5 and the other BMPs are probably numerous. Although the neurotrophic actions of these proteins on certain neuronal populations have been documented (Krieglstein et al. 1995a, 1995b; Farkas et al. 1997, 1999; Jordan et al. 1997; Sullivan et al. 1997, 1999; Galter et al. 1999; Strelau et al. 2000; Reiriz et al. 2002; Hurley et al. 2004) and the expression of BMPR-II (Charytoniuk et al. 2000) and GDF-15 (Schober et al. 2001) has been shown to be up-regulated following CNS injury, the precise roles of these proteins in the mature CNS remain to be established.

5.4.4 GDF-5 expression in the developing rat brain

The present study demonstrates a temporally-regulated expression of GDF-5 protein in the developing rat brain, suggesting a function for this protein during brain maturation. GDF-5 binds to the receptors BMPR-IB and BMPR-II (Nishitoh et al. 1996), both of which have been shown to be widely expressed from E12 onwards in the mouse brain (Dewulf et al. 1995; Zhang et al. 1998). The present study found that GDF-5 expression in the rat brain begins on E12, peaks at E14 and persists throughout the embryonic developmental period. This finding, combined with the presence of its receptors during this period, implies that GDF-5 plays a role in brain development. Other members of the BMP family have been shown to play many roles during neural development, in various brain regions (for review see Mehler et al. 1997). In particular, BMPs have been found to promote a commitment to astroglial cell lineage (Gross et al. 1996; Mabie et al. 1997); this is supported by the appropriate temporal expression of the BMPs and their receptors in the developing brain (Soderstrom et al. 1996; Zhang et al. 1998). Furthermore, BMP-2 can alter the developmental pathway of embryonic mouse brain cells from neurogenesis to gliogenesis *in vitro* (Nakashima et al. 2001; Yanagisawa et al. 2001). A recent study found that transgenic over-expression of BMP-4 increased astroglial and decreased oligodendrocyte lineage commitment in multiple brain regions (Gomes et al. 2003). However, the effects of BMPs may be both spatially and temporally dependent. For example, BMPs can induce neuronal differentiation of ventricular zone progenitor cells (Li et al. 1998, Mabie et al. 1999), while they promote astroglial cell lineage in subventricular zone progenitors (Gross et al. 1996). The BMPs have also been suggested to be involved in cerebellar development, since various BMPs have been shown to induce the expression of granule neuron markers *in vitro* (Alder et al. 1999) and caBMP receptors can cause cerebellar abnormalities in the chick embryo

(Ming et al. 2002). The role(s) that GDF-5 plays during normal brain development remain to be established. One possibility is that the GDF and BMP family members play opposing or synergistic roles in the determination of cell fate in the CNS.

5.4.5 GDF-5 expression in the developing rat ventral mesencephalon

After finding GDF-5 expression in the adult rat nigrostriatal system, its expression in the region of the brain from which this system develops, the VM, was investigated. Transplantation of embryonic VM tissue has been used as an experimental therapy for Parkinson's disease (for recent reviews, see Hagell and Brundin 2001, Barker 2002, Borlongan and Sanberg 2002). However, one of the limiting factors of this approach is the poor survival of the dopaminergic neurons after transplantation. Supplementation of the transplants with various neurotrophic factors has had some success in improving neuronal survival (for review, see Brundin et al. 2000). Transplantation of rat VM tissue to 6-OHDA lesioned adult rats is used to model the clinical situation, in order to optimise transplantation procedures and to experimentally test factors that may improve graft survival. In the rat VM, dopaminergic neurons proliferate between E11 and E15, undergoing their final mitotic divisions on E14-15 (Hanaway et al. 1971; Lauder and Bloom 1974; Altman and Bayer 1981; Solberg et al. 1993; Bayer et al. 1995). Thus, tissue is generally harvested from the rat VM at E14 for experimental modelling of transplantation for Parkinson's disease. The identification of neurotrophic factors that are expressed in the developing VM at the time that this tissue would be harvested for transplantation should yield important information about potential factors which could be used to support this tissue after transplantation.

The expression of various BMPs (Jordan et al. 1997) in the developing rodent mesencephalon suggest that the BMPs play a role in the development and survival of these nigrostriatal dopaminergic neurons. The neurotrophic abilities of various BMPs

and GDFs on dopaminergic neurons from the E14 VM have been documented (Krieglstein et al. 1995a, 1995b; Jordan et al. 1997; Sullivan et al. 1998; Strelau et al. 2000; Brederlau et al. 2002). The present study shows for the first time that GDF-5 is expressed in the developing VM from E12 onwards and that levels of this protein reach a peak on E14, the day on which dopaminergic neurons undergo their terminal differentiation. Neurons of the rat VM are produced between days E11 and E15, with maximal production being reported on days E14 and E15 (Hanaway et al. 1971) or on E13 (Lauder and Bloom, 1974). A further study found that production of these neurons was evenly distributed throughout days E13, 14 and 15 (Altman and Bayer 1981). Dopamine can be first detected in the tegmentum of rat embryos of crown-rump length 9 mm, which corresponds to day E13 (Olson and Seiger 1972). Thus the time-course of GDF-5 expression found in the present study parallels that of VM dopaminergic neuron production, suggesting that it plays a role in dopaminergic neuron development. Furthermore, the time-course of GDF-5 expression in this brain region was found to be consistent with that of TH, a marker for dopaminergic neurons. These data, along with previous reports that administration of exogenous rhGDF-5 has survival-promoting effects on dopaminergic neurons in cultures of rat E14 VM (Krieglstein et al. 1995b; Lingor et al. 1999), further supports the use of GDF-5 as an adjunct to E14 VM transplants for Parkinson's disease. It is also interesting to note that the expression of Nurr1, an orphan nuclear receptor crucial for the development of midbrain dopaminergic neurons (Zetterstrom et al. 1997; Castillo et al. 1998), during development of the rat VM, peaks between E13 and E15 (Volpicelli et al. 2004), paralleling that of GDF-5. It is possible that GDF-5 may play a role in Nurr1 expression and subsequently TH expression (see Chapter 9).

5.4.6 GDF-5 expression in the developing rat striatum

GDF-5 was strongly expressed in the embryonic rat striatum at each of the embryonic ages examined. Given the strong expression of GDF-5 at E14, it is likely that protein expression begins at an earlier stage in development than this, however earlier ages were not examined in this study. The demonstration that various BMPs and their receptors, including those for GDF-5, have been detected in the striatum at various stages during development (Dewulf et al. 1995; Soderstrom et al. 1996; Mehler et al. 1997; Zhang et al. 1998; Charytoniuk et al. 2000), suggests that various BMPs/GDFs may play a role in the striatal development. The role(s) of GDF-5 in the striatal development may be diverse. In the rat, axons of nigral dopaminergic neurons start to invade the striatum at E16 and continue to do this pre- and post-natally (Unsicker et al. 1996). This process of neurite outgrowth from the VM to the striatum has been shown to be controlled by spatially and temporally regulated chemoattractive (from the MFB and later the striatum) and chemoreplusive (from the brainstem and cortex) cues, from the surrounding brain regions (Gates et al. 2004). It is possible that GDF-5 may act as a chemoattractant for developing nigrostriatal axons.

The present study also found GDF-5 expression in the developing and adult rat striatum. The striatum contains GABAergic neurons, a subset of which co-express the calcium binding protein calbindin, that degenerate in Huntingtons disease (Kiyama et al. 1990). As BMP-2 has been shown to promote calbindin phenotype and promote dendritic growth in cultured rat striatal neurons (Gratacòs et al. 2001), it is possible GDF-5 may also exert an effect on this neuronal population, and may be useful to study in relation to its use a neurotrophic factor for treatment of Huntingtons disease.

Figure 5.1:

Representative immunoblots of (A) cell extracts and (B) conditioned medium from a GDF-5 secreting CHO cell line, stained with P3/66 antibody. (A) shows that intracellularly the P3/66 antibody detects a number of high molecular weight bands but not the mature 15kDa processed form of GDF5. (B) shows conditioned medium from GDF-5 secreting CHO cells, lane1 = control transfected CHO cells at 1 DIV, lane 2= control cells at 3 DIV, lanes 3-8 = GDF-5 secreting cells at 1, 3, 5, 7, 14, 21 DIV respectively. Lane 9 = 50ng rhGDF-5 run as a positive control. Only the mature form of GDF-5 (15kDa) was detected in conditioned medium from the GDF-5 secreting CHO cells.

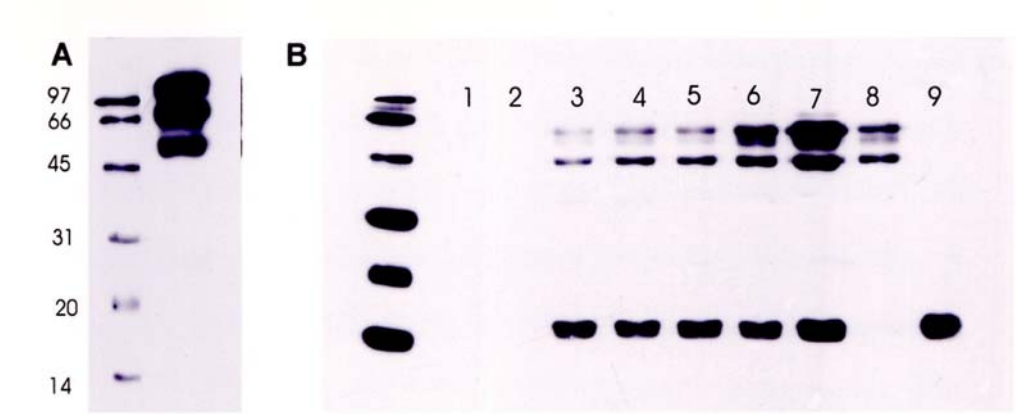


Figure 5.2:

(A) Clustal W sequence alignment showing the residues in the C-terminal end of the human GDF-5 protein that the antibody P3/66 recognises. Alignments were done using a web-based version of Clustal W available at www.embl.org.

(B) Representative western blot showing characterisation of the P3/66 antibody. 50ng/ml rhGDF-5 was run in each lane, in either reducing sample buffer containing 100mM of DTT (+ DTT) or in sample buffer not containing DTT (-DTT). When run under reducing conditions which breaks the di-sulphide bonds involved in dimer formation, rhGDF-5 migrates as a monomer of approximately 15kDa. Although the antibody shows greater affinity for the reduced form of rhGDF-5, the 25kDa non-reduced form of GDF-5 can be detected under non-reducing conditions.

A

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GDF5 MRLPKLLTFLLWYLAWLDLEFICTVLGAPDLGQRPQGTRPGLAKAEAKERPPLARNVFRP 60

GDF5 GGHSYGGGATNANARAKGGTGQTGGLTQPKKDEPKKLPPRPGGPEPKPGHPPQTRQATAR 120

GDF5 TVTPKGQLPGGKAPPKAGSVPSSFLLKKAREPGPPREPKEPFRPPPITPHEYMLSLYRTL 180

GDF5 SDADRKGGNSSVKLEAGLANTITSFIDKGQDDRGPVVRKQRYVFDISALEKDGLLGAELR 240

GDF5 ILRKKPSDTAKPAAPGGGRAAQLKLSSCPSGRQPASLLDVRSVPGLDGSGWEVFDIWKLF 300

GDF5 RNFKNSAQLCLELEAWERGRAVDLRGLGFDRAARQVHEKALFLVFGRTKKRDLFFNEIKA 360

GDF5 RSGQDDKTVYEYLFSQRRKRRAPLATRQGKRPSKNLKARCSRKALHVNFKDMGWDDWIIA 420
P3/66                        PLATRQGKRPSKNLKARC
GDF5 PLEYEAFHCEGLCEFPLRSHLEPTNHAVIQTLMNSMDPESTPPTCCVPTRLSPISILFID 480

GDF5 SANNVVYKQYEDMVVESCGCR 501
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B

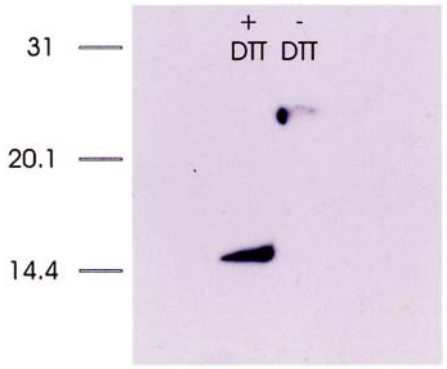


Figure 5.3:

Representative western blots of an immunoabsorption assay confirming the specificity of P3/66 antibody for GDF-5. (A) represents a control blot where the primary antibody was applied as normal. (B) shows a blot probed with P3/66 antibody that had been preincubated with excess monomeric rhGDF-5 prior to application on the blot. Lane 1 = adult rat striatum, lane 2 = adult rat SN, lane 3 = 50ng rh GDF-5.

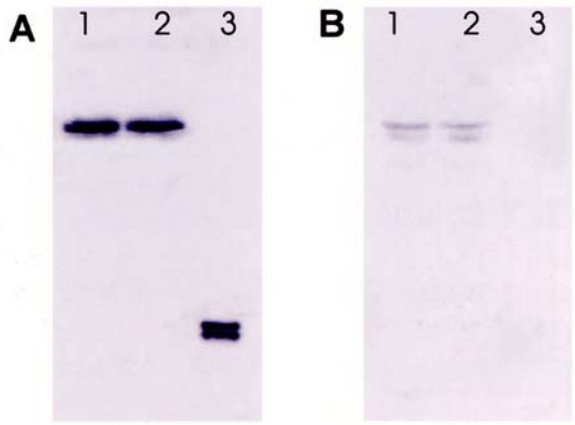


Figure 5.4:

Representative western blot showing two different processed forms of the GDF-5 precursor protein in adult rat brain samples. Values shown on the left indicate molecular weight in kDa. Lane 1 = E20 VM, lane 2 = adult rat striatum, lane 3 = adult rat SN. The two different processed forms are indicated by black arrows.

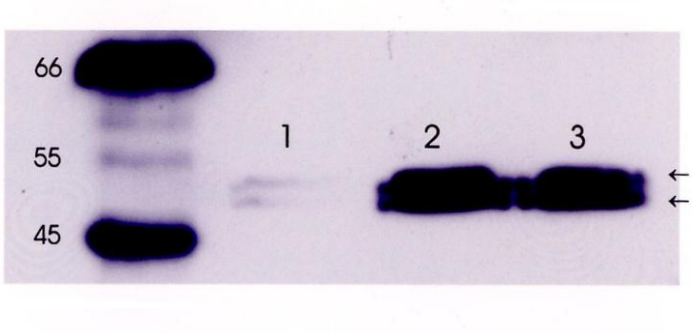


Figure 5.5:

(A) Representative immunoblot of GDF-5 expression in whole brain extracts from rats aged E11 to E20, P1, 6, 14, 22 and in the adult rat striatum (STR), whole brain (WB) and kidney (KID) using the P3/66 antibody. The bands at 55kDa correspond to the predicted molecular weight of the GDF-5 precursor protein. Molecular weight markers are shown in the left hand lane. RhGDF-5 was run in the right hand lane. The blot was stripped of GDF-5 antibody and reprobed with antibody to β-actin (B).

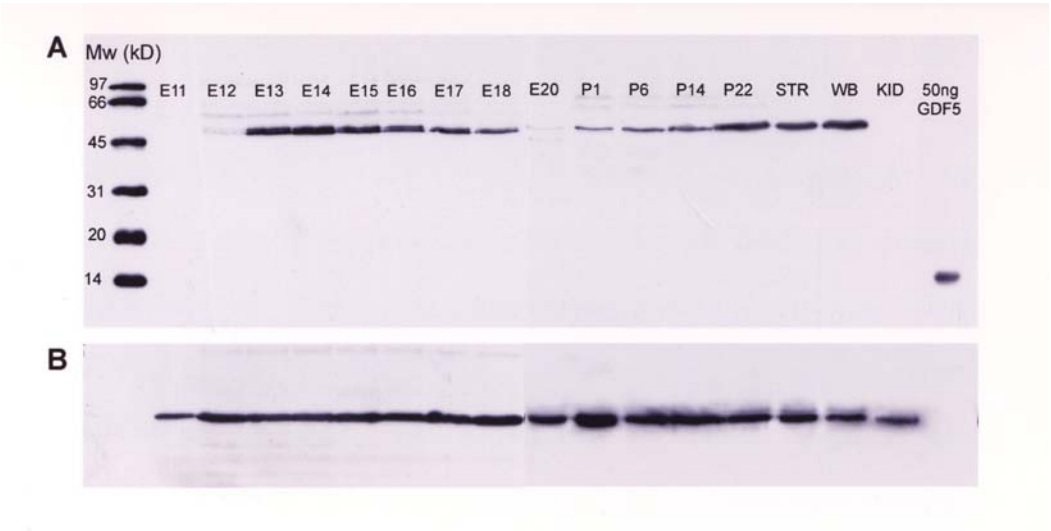


Figure 5.6:

(A) Representative immunoblot of GDF-5 expression in extracts from adult rat tissues using the P3/66 antibody to GDF-5. Lane 1 = brain, lane 2 = heart, lane 3 = lung, lane 4 = liver, lane 5 = kidney, lane 6 = spleen. Molecular weight markers are shown in the left hand lane. rhGDF-5 was run in the right hand lane (data not shown). The blot was stripped of GDF-5 antibody and reprobed with antibody to β-actin (B).

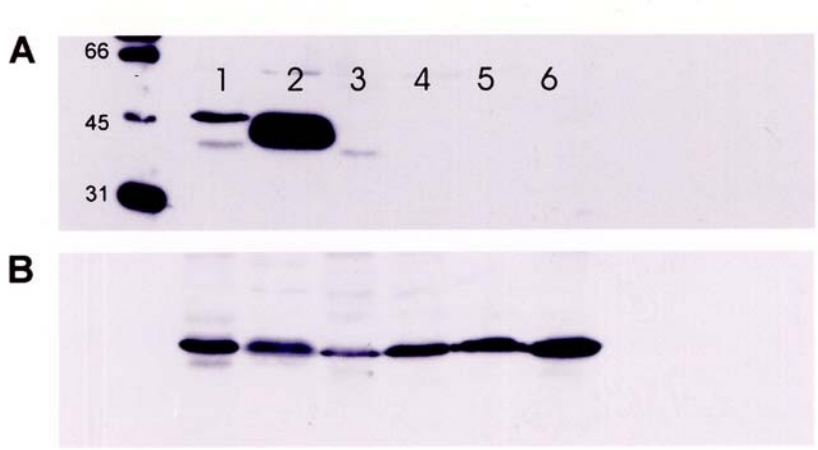


Figure 5.7:

(A) Representative immunoblot of GDF-5 expression in extracts from adult rat brain using the P3/66 antibody. Lane 1 = cortex, lane 2 = striatum, lane 3 = midbrain, lane 4 = cerebellum. The blot was stripped of GDF-5 antibody and reprobed with antibody to β-actin (B).

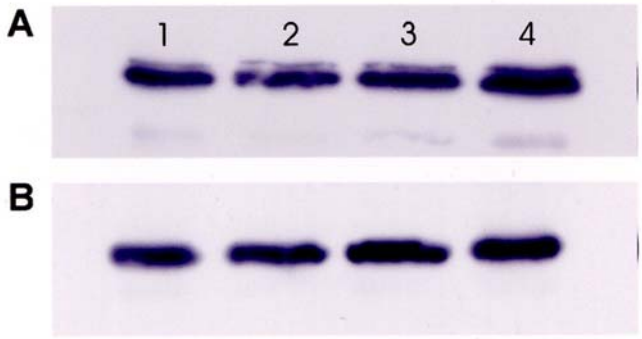


Figure 5.8:

GDF-5 expression in the developing rat VM *in vivo*. (A) Representative immunoblot of GDF-5 (55 kDa) expression in VM from rats aged E12, 13, 14, 15, 18, 19 and P2. Blots were stripped of GDF-5 antibody and probed with antiserum to TH (B; bands at 45 kDa), then stripped and probed with antibody to β-actin (C).

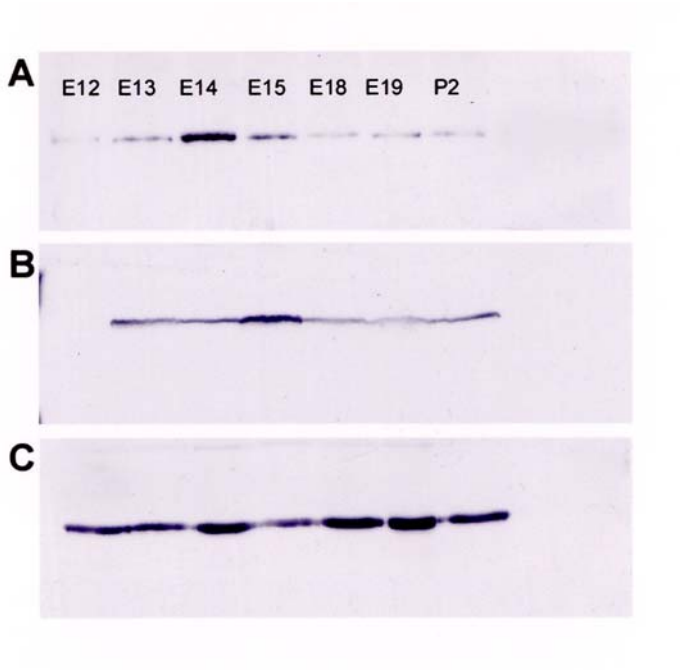


Figure 5.9:

Immunocytochemical analysis of GDF-5 expression in cultures of E14 rat VM after 2 DIV, using P3/66 antibody, which was visualised using anti-mouse IgG linked to FITC (A). Cultures were counter-stained with DAPI (B). (Bar = 50 μm.)

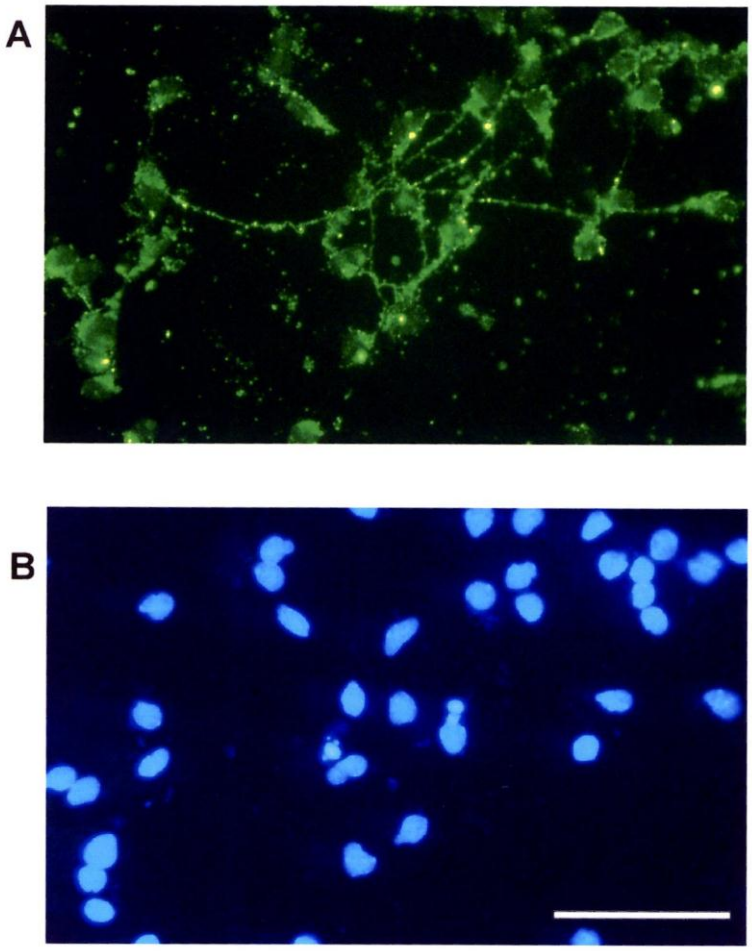
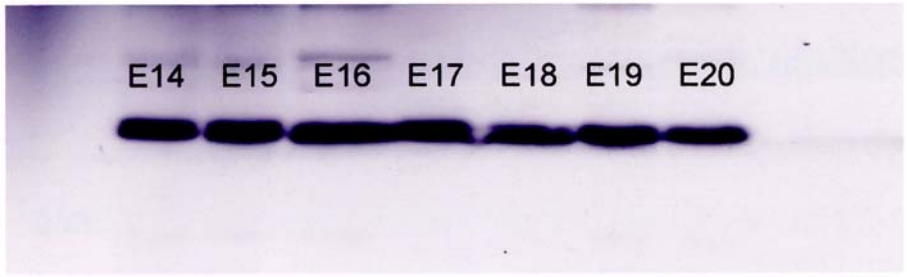


Figure 5.10:

GDF-5 expression in the developing rat striatum *in vivo*. Representative immunoblot of GDF-5 (55 kDa) expression in the striatum from rats aged E14-E20.



6.0 EFFECTS OF GDF-5 ON THE NUMBERS AND MORPHOLOGY OF EMBRYONIC RAT MIDBRAIN DOPAMINERGIC NEURONS *IN VITRO*

6.1 Aims

- To examine the effects of GDF-5 treatment on numbers of dopaminergic neurons and astrocytes in cultures of E14 rat VM.
- To examine the expression of BMP receptors in E14 rat VM and potential changes in their expression with time in culture.
- To determine if BMPR-Ib is necessary for GDF-5 to have effects on dopaminergic neurons *in vitro*.
- To examine the effects of GDF-5 on the morphology of dopaminergic neurons in cultures of E14 rat VM.

6.2 Introduction

The TGF-β superfamily is a large family of structurally related growth factors. It can be divided into two main groups, based on phylogenetic relationships; the TGF-β/Act group and the BMP/GDF group (for recent review, see de Caestecker, 2004 and Chapter 3). The BMPs represent one of the largest families of the TGF-β superfamily and have been shown to have effects on a variety of neuronal populations (for reviews, see Mehler et al., 1997; Ebendal et al, 1998). Various BMPs, as well as their receptors (BMPRs) and antagonists, have been shown to be expressed in the adult rat brain, including the SN and the striatum, suggesting that BMPs may modulate the function of these regions in adulthood (Tomizawa et al., 1995; Soderstrom et al., 1996; Mehler et al., 1997; Zhang et al., 1998; Soderstrom & Ebendal, 1999; Charytoniuk et al., 2000; Strelau et al., 2000; Chen et al., 2003). BMP-2, -4 and -6 are expressed in the developing rat VM at E13 and E15 (Jordan et al., 1997). Recently, it has been reported that BMP-5, -6 and -7 increase the numbers of dopaminergic neurons in cultures of E14 rat VM (Brederlau et al., 2002). These studies suggest that BMP family members may be involved in dopaminergic neurogenesis and / or provide neurotrophic support in the developing VM.

The GDFs belong to the same subfamily of the TGF-β superfamily as the BMPs and have been shown to be involved in a variety of developmental processes, ranging from chondrogenesis in the developing limb to the regulation of ovarian follicle development (for recent reviews, see Edwards & Francis-West, 2001; Balemans & Van Hul, 2002; Knight & Glister, 2003). The role of GDF-5 in limb development has been well established (for review see Buxton et al., 2001) and mutations in the GDF-5 gene result in skeletal abnormalities in mice (Storm et al., 1994) and humans (Thomas et al., 1996). Recently it has emerged that a number of the GDFs are expressed in the nervous system (Lee, 1991; Bottner et al., 1999; Nakashima et al., 1999; Soderstrom & Ebendal, 1999;

Zhao et al., 1999; Vokes & Kreig, 2000; Schober et al., 2001; Watabake et al., 2001). We (O'Keeffe et al, 2004a; see Chapter 5) and others (Krieglstein et al., 1995b) have shown that GDF-5 is expressed in the developing rat brain. Furthermore, we have found that GDF-5 is expressed in the developing rat VM during the period of dopaminergic neurogenesis, which peaks on E14 (O'Keeffe et al. 2004a; see Chapter 5). It has been shown that GDF-5 can protect E14 rat VM dopaminergic neurons from neurotoxic agents (Krieglstein et al., 1995a; Lingor et al., 1999). Furthermore, GDF-5 has neuroprotective effects on the adult nigrostriatal dopaminergic pathway in the 6-OHDA rat model of PD (Sullivan et al., 1997, 1999; Hurley et al., 2004) and improves the survival of grafts of E14 rat dopaminergic neurons in this rat model of PD (Sullivan et al., 1998). Together, these studies suggest that GDF-5 may act as a dopaminergic neurotrophin, both during the development of the VM and in the adult brain. Although GDF-5 has been previously shown to increase the survival of dopaminergic neurons *in vitro* (Krieglstein et al., 1995b), its effects on the morphological development of these neurons has not been reported.

6.3 Results

6.3.1 GDF-5 increases the numbers of dopaminergic neurons and astrocytes in E14 rat VM cultures

Treatment of E14 rat VM cultures for 6 DIV with GDF-5 exerted effects on dopaminergic neurons and astrocytes, since Western blotting experiments showed that GDF-5 treatment increased the levels of expression of TH and GFAP protein in these cultures. However GDF-5 did not alter the expression of the neuronal marker β-III tubulin (Figure 6.1A-D).

GDF-5 treatment resulted in an increase in the numbers of TH-immunopositive neurons surviving for 6 DIV (Figure 6.2A, B, C). Application of GDF-5 at doses of 1ng/ml and 10ng/ml resulted in significant increases (3.1- and 2.6-fold, respectively) in the number of dopaminergic neurons, when compared to control values (Figure 6.4A; $P<0.001$). No significant difference was observed between the numbers of dopaminergic neurons in cultures treated with 1ng/ml and 10ng/ml GDF-5.

GDF-5 treatment for 6 DIV also induced a marked increase in the numbers of GFAP-immunopositive cells in the cultures (Figure 6.3A, B, C). Treatment with GDF-5 at doses of 1ng/ml and 10ng/ml resulted in significant increases (2.3- and 5.6-fold, respectively) in the number of astrocytes, when compared to control values (Figure 6.4B; $P<0.001$). In contrast to GDF-5's effect on the numbers of dopaminergic neurons, its effect on astrocytes was dose-dependent, with a significant difference observed between the numbers of GFAP-immunopositive cells in cultures which had been treated with 1ng/ml and 10ng/ml GDF-5 (Figure 6.4B; $P<0.001$).

There was no significant difference in either the total number of cells (as determined by DAPI staining) or the number of TUJ-1-immunopositive cells between any of the treated or untreated cultures (see Chapter 7, Figure 7.4).

6.3.2 Loss of BMPR-Ib expression in vitro abolishes the ability of GDF-5 to increase the numbers of dopaminergic neurons

RT-PCR analysis demonstrated the expression of all three BMP receptors, BMPR-Ia, -Ib and -II, in extracts of E14 rat VM (Figure 6.5A). However, when cells from the E14 VM were cultured, the expression of BMPR-Ib was dramatically down-regulated, even after 1 DIV (Figure 6.5C). Low levels of BMPR-Ib mRNA were present at 1, 2 and 4 DIV (Figure 6.5C), but expression was completely lost by 6 DIV (Figure 6.5B, C). In contrast, the expression of BMPR-Ia and –II remained in the cultures after 6 DIV (Figure 6.5B). Immunocytochemistry was used to confirm the RT-PCR. Cells which were immunopositive for BMPR-II and BMPR-Ia were detected in the cultures after 6 DIV (Figure 6.6A and B, respectively), but no positive immunostaining for BMPR-Ib was observed in any cultures at this time-point (Figure 6.6C). Negative controls where the primary antibody was omitted displayed no staining for each antibody tested (Figure 6.6D). Cultures treated with 10ng/ml GDF-5 for 24 h at the time of plating displayed a significant increase in the numbers of dopaminergic neurons when compared to untreated cultures (Figure 6.7A; $P<0.05$). However, when 10ng/ml GDF-5 was added at 6 DIV for 24 h, no increase in the numbers of dopaminergic neurons was observed (Figure 6.7B).

6.3.3 GDF-5 improves the morphology of dopaminergic neurons in E14 rat VM cultures

Morphological analysis of the cultured dopaminergic neurons revealed that treatment with GDF-5 (at doses of either 1ng/ml or 10ng/ml) resulted in increased differentiation of these cells *in vitro*. Dopaminergic neurons had a more complex morphology following GDF-5 treatment when compared to controls (Figure 6.8A, B).

A significant increase in total neurite length per dopaminergic neuron was observed in GDF-5-treated cultures, when compared to control cultures (Figure 6.9A; $P<0.001$). Both doses of GDF-5 significantly increased neurite length at all levels of the neuritic field ($P<0.001$), with the greatest increases (at least four-fold) measured in the length of secondary and tertiary neurites and smaller increases observed in the length of primary and quaternary neurites (Figure 6.10A). The average somal area per dopaminergic neuron was also significantly increased following GDF-5 treatment (Figure 6.9B; $P<0.001$). The number of branch points per dopaminergic neuron (as measured by the mean number of nodes per dopaminergic neuron) was significantly increased by GDF-5 treatment (Figure 6.9C; $P<0.001$). Analysis of the degree of branching at different levels of the neuritic field established that the greatest increases were at the level of secondary and tertiary branches, compared to those at primary and quaternary branches (Figure 6.10B). There was no significant difference between the effects of the two doses of GDF-5 on any of these morphological parameters.

6.4 Discussion

6.4.1 Effects of GDF-5 on the cellular composition of E14 rat VM cultures

This study examined the effect of GDF-5 on cultures of E14 rat VM. When applied at the time of plating, GDF-5 increased the numbers of dopaminergic neurons present in the cultures after 6 DIV, in agreement with previous studies (Krieglstein et al., 1995b). GDF-5 was added to the culture medium at this early stage because it had been previously observed that during the development of the rat VM, GDF-5 protein levels peak on E14 (O'Keeffe et al. 2004a; see Chapter 5). It is known that dopaminergic neurons in the rat VM undergo their final mitosis at E14 or E15 (Altman & Bayer, 1981; Bayer et al., 1995), thus factor(s) involved in the induction of a dopaminergic fate or in regulating the survival of these neurons would be expected to peak in the VM at about E14.

Previous reports have shown that addition of the neurotrophic factors GDNF, TGF-β1/2/3 or ActA, to E14 rat VM cultures at 24 hs after plating, induced 1.5-, ~2.3- and 1.6-fold increases, respectively, in the numbers of dopaminergic neurons after 8 DIV when compared to controls (Krieglstein et al., 1995a). It has also been shown that treatment of E14VM cultures with 10ng/ml BMP-2 for 7DIV, resulted in a 2.37 fold increase in the numbers of dopaminergic neurons compared to controls (Reiriz et al. 1998). In the present study, GDF-5 treatment from the time of plating resulted in a ~3-fold increase in dopaminergic neuronal survival after 6 DIV. Thus, GDF-5 has powerful neurotrophic effects on mesencephalic dopaminergic neurons in culture and is one of the most potent dopaminergic neurotrophins documented to date.

It has recently been reported that GDF-5 treatment does not increase the numbers of dopaminergic neurons in cultures of E14 rat VM (Brederlau et al., 2002). The apparent discrepancy between this study and the present one may be explained by the

fact that, in our study, GDF-5 was added from the time of plating of the cultures (i.e. DIV 0), whereas Brederlau and co-workers did not apply GDF-5 to their cultures until DIV 7. Given that GDF-5 protein levels in the developing rat VM peak on E14 and are subsequently down-regulated, reaching lowest levels around birth (O'Keeffe et al., 2004a; see Chapter 5), it is possible that E14 dopaminergic neurons may become less responsive to GDF-5 as they mature. This may be due to changing receptor expression patterns with maturation *in vitro* (see below).

In agreement with previous reports (Krielgstein et al., 1995b; Brederlau et al., 2002), it was found here that GDF-5 treatment resulted in an increase in the numbers of GFAP-positive cells in these cultures. This raises the possibility that GDF-5 may mediate its effect on neuronal survival in the cultures indirectly, by increasing the numbers of astrocytes and thus increasing the levels of astrocyte-derived neurotrophic factors. It has been shown that the presence or absence of astrocytes affects the morphological development of E14 rat VM dopaminergic neurons *in vitro* (Wood et al., 2003). Thus, GDF-5 may indirectly increase the numbers of dopaminergic neurons and improve their morphological development by increasing the numbers of astrocytes present in the culture. The effect of GDF-5 to increase the number of astrocytes in these cultures was dose-dependent, unlike its effect on the number of dopaminergic neurons. Thus, the increase in the number of astrocytes with increasing dose of GDF-5 was not paralleled by an increase in the numbers of dopaminergic neurons. This suggests that the effect of GDF-5 on these neurons is not solely due to an astrocyte-mediated action. Indeed, another study in our laboratory has found that the effects of GDF-5 on the survival of dopaminergic neurons *in vitro* are not significantly reduced by depletion of astrocytes in E14 rat VM cultures (Wood et al., in preparation). This is in agreement with a previous study showing that although BMP-2 increases the numbers of astroglial cells in E14 rat VM cultures, its neurotrophic effect on dopaminergic neurons is not

mediated by astroglial cells (Reiriz et al. 1998). A direct action on dopaminergic neurons is substantiated by the fact that GDF-5 application to E14 rat VM cultures at 1 DIV results in nuclear accumulation of phosphorylated smad proteins in these cells (see Chapter 7). The possibility of a direct action of GDF-5 on dopaminergic neurons is also supported by the fact that mRNA for both BMPR-Ib and BMPRII, the receptors which are necessary for GDF-5 signalling (Nishitoh et al. 1996), have been found in the adult rat SN and striatum (Chen et al., 2003). Furthermore, the expression of BMPR-II mRNA has been localised to dopaminergic neurons in the adult rat SN (Charytoniuk et al., 2000).

6.4.2 Expression of BMP receptors in the rat VM

In agreement with previous studies on chick embryos (Ming et al., 2002), we have found BMPR-II, -Ia and BMPR-Ib are expressed in the rat VM at E14. The expression of various BMPs (Jordan et al., 1997), GDF-1 (Wall et al. 2000) and GDF-5 (O'Keeffe et al., 2004a; see Chapter 5) in the VM at this stage suggests that cells in this tissue at this stage of development are responsive to BMP and GDF signalling. It has been reported that the expression of BMPs generally peaks around the perinatal period (for review, see Mehler et al., 1997). However, we have found that GDF-5 expression peaks much earlier during rat brain development (at E14 in both whole brain and VM), reaching its lowest level around the perinatal period before being up-regulated in the post-natal period to reach high levels in the adult brain, including the SN and striatum (O'Keeffe et al., 2004a; see Chapter 5). The expression of BMPR-II, -Ia and BMPR-Ib in the adult rat striatum and SN (Chen et al. 2003), suggests that BMPs/GDF-5 play roles in these regions in the adult rat brain.

It has been suggested that the maturation state of dopaminergic neurons in E14 VM cultures at 7 DIV are representative of these mature dopaminergic neurons at the

time of birth (Brederlau et al., 2002). Brederlau and co-workers have shown that neurons at this stage of development are affected by BMP signalling, as evidenced by the increase in TH-immunopositive neurons and nuclear translocation of smad proteins, but that they are largely unresponsive to GDF-5 (Brederlau et al., 2002). Our findings complement these results, since we show that the expression of BMPR-Ib is down-regulated in these cultures after 6 DIV, suggesting that they become unresponsive to GDF-5 at this stage in culture. In agreement, we found that addition of GDF-5 for 24 h at 0 DIV (when BMPR-Ib is present) induced a significant increase in the numbers of dopaminergic neurons in these cultures, whereas addition of GDF-5 for 24 h after 6 DIV (when BMPR-Ib is no longer expressed) did not. This suggests that the actions of GDF-5 and various other BMPs on the development and survival of rat VM dopaminergic neurons may be exerted at different stages during embryonic development, with GDF-5 acting at an earlier stage than the BMPs. Given its earlier expression, GDF-5 may be involved in the induction of a dopaminergic phenotype in progenitor cells in this area of the brain, an action which has been bestowed on other members of the TGF-β superfamily (Farkas et al., 2003, see Chapter 6).

6.4.3 Effects of GDF-5 on the morphology of dopaminergic neurons

Having shown that GDF-5 increases the numbers of dopaminergic neurons *in vitro*, it was assessed whether this factor might also affect the morphological development of these neurons *in vitro*. Previous studies have shown that BMPs can affect the morphological development of striatal neurons (Gratacòs et al., 2001). Another member of the TGF-β superfamily, GDNF, has been shown to be a potent neurotrophic factor for dopaminergic neurons in VM cultures (Lin et al., 1993; Krieglstein et al., 1995a). It has been reported that treatment with 10ng/ml GDNF for 7 DIV resulted in an increase in neurite length and branching of dopaminergic neurons in E14 rat VM cultures (Widmer

et al., 2000). In that study, GDNF resulted in a 1.5-fold increase in the total neurite length per dopaminergic neuron (Widmer et al., 2000). Also, treatment of E14 VM dopaminergic neurons for 5DIV with 10ng/ml persphin has been shown to result in a 2-fold increase in total neurite length per dopaminergic neurons (Åkerud et al. 2002). The present findings are similar, showing that GDF-5 treatment for 6 DIV resulted in a 2.5-fold increase in the total neurite length per dopaminergic neuron. Cell somal area was reported to be increased by 1.3-fold by GDNF treatment in the Widmer study, while in the present study a 1.4-fold increase in this parameter due to GDF-5 treatment was observed. Widmer and co-workers also found that treatment with GDNF resulted in a 1.4-fold increase in the number of primary nodes per dopaminergic neuron (Widmer et al., 2000). In agreement, GDF-5 treatment resulted in a 1.4-fold increase in the numbers of primary nodes per dopaminergic neuron. BMP-2 has also been shown to affect the morphological development of E14 rat VM dopaminergic neurons (Reiriz et al. 1998). In agreement with the present findings, treatment of rat E14 VM cultures with 10ng/ml BMP-2 for 7 DIV resulted in a 1.4-fold increase in the number of primary nodes per dopaminergic neuron, although no effect on somal area was seen (Reiriz et al. 1998). Furthermore, our detailed morphological analysis showed that GDF-5 treatment resulted in an increase in neurite branching and neurite length at all levels of the neuritic field. The greatest effects were seen on the secondary and tertiary neurites, where GDF-5 induced a ~5-fold increase in length (in comparison to ~1.6-fold increase in length of primary neurites).

These results show that GDF-5, like other members of the TGF-β superfamily, has potent effects on the morphological development of VM dopaminergic neurons *in vitro*. The observed increases in dopaminergic neuronal survival and in the size of their neuritic fields have important implications for research on intrastriatal neuronal transplantation for PD, where efforts are concentrated on promoting the survival of

dopaminergic neurons and their functional integration into the host striatum after transplantation. It has been reported that GDNF treatment can increase fiber outgrowth from embryonic rat dopaminergic VM tissue after transplantation into the striatum of 6-OHDA lesioned rats (Rosenblad et al., 1996; Wang et al., 1996). It has also been shown that GDF-5 is at least as effective as GDNF in improving the survival of grafts of E14 VM in a rat model of PD (Sullivan et al., 1998). Furthermore previous studies have found that during the development of the rat VM, GDF-5 expression peaks at E14 (O'Keeffe et al., 2004a; see Chapter 3), while that of GDNF peaks at a later date (O'Keeffe and Sullivan, unpublished observations). These findings suggest that GDF-5 may be a candidate neurotrophic factor for use in attempts to improve the survival, neurite outgrowth and functional integration of transplanted embryonic dopaminergic neurons for the treatment of PD.

Figure 6.1:

Representative Western blots on protein extracts from cultures of E14 rat VM grown for 6 DIV, immunostained for (A) TUJ, (B) GFAP, (C) TH and (D) β-actin. Densitometric analysis after normalisation to β-actin levels for the corresponding sample showed that GDF-5 treatment induced no change in TUJ levels but increased levels of GFAP and TH.

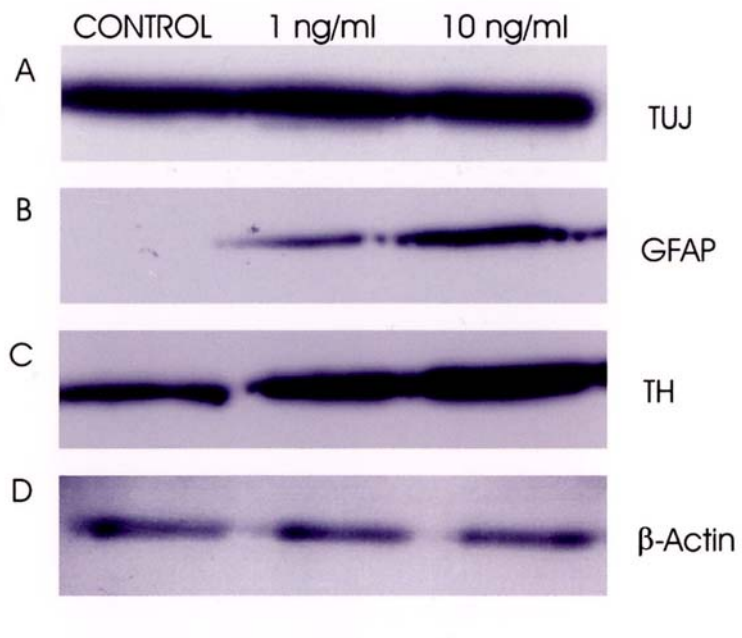


Figure 6.2:

Photomicrographs showing representative cultures of E14 rat VM grown for 6 DIV, immunocytochemically stained for TH. (A) shows a control culture, (B) shows a culture that received 1ng/ml GDF-5 every 2 DIV, (C) shows a culture that received 10ng/ml GDF-5 every 2 DIV.

Scale bar = 100μm

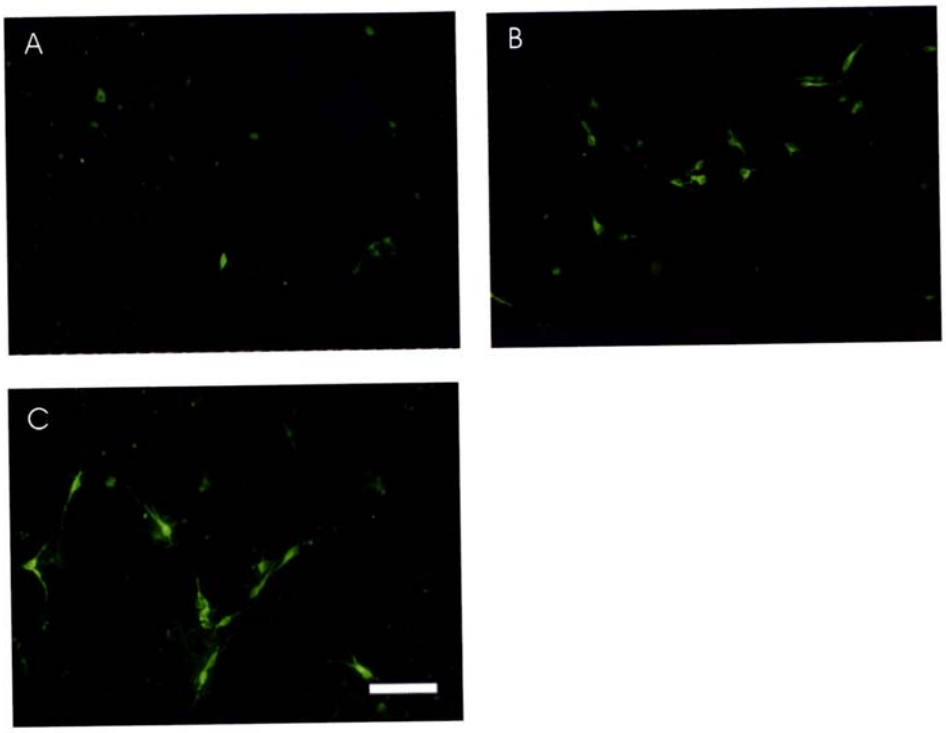


Figure 6.3:

Photomicrographs showing representative cultures of E14 rat VM grown for 6 DIV, immunocytochemically stained for GFAP. (A) shows a control culture, (B) shows a culture that received 1ng/ml GDF-5 every 2 DIV, (C) shows a culture that received 10ng/ml GDF-5 every 2 DIV.

Scale bar = 100μm

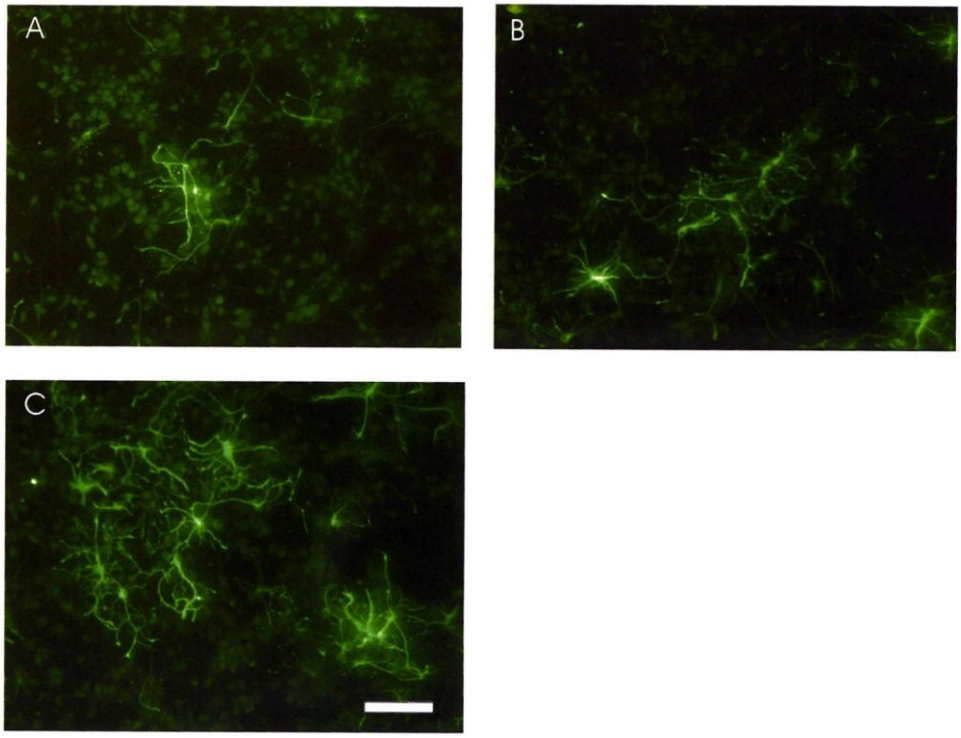


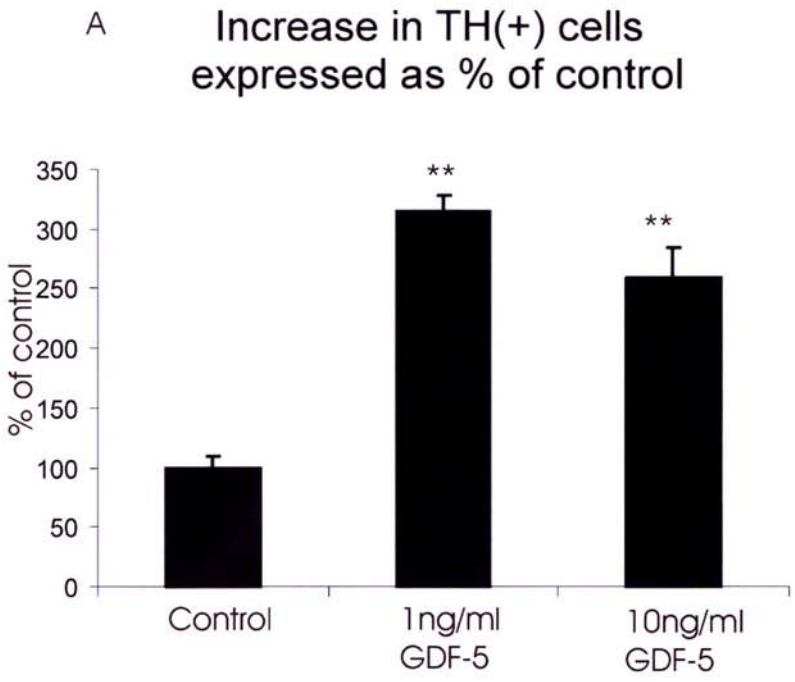
Figure 6.4:

Increase in numbers of (A) TH-immunopositive and (B) GFAP-immunopositive cells in cultures treated with 1ng/ml or 10ng/ml GDF-5 every 2DIV for 6 DIV, expressed as a percentage of those in untreated cultures. Data represent mean ± SEM taken from three independent experiments.

** $P<0.001$ compared to untreated (control) cultures

$ $P<0.001$

A


Increase in TH(+) cells
expressed as % of control
% of control
350
300
250
200
150
100
50
0
**
**
Control
1ng/ml
GDF-5
10ng/ml
GDF-5

B

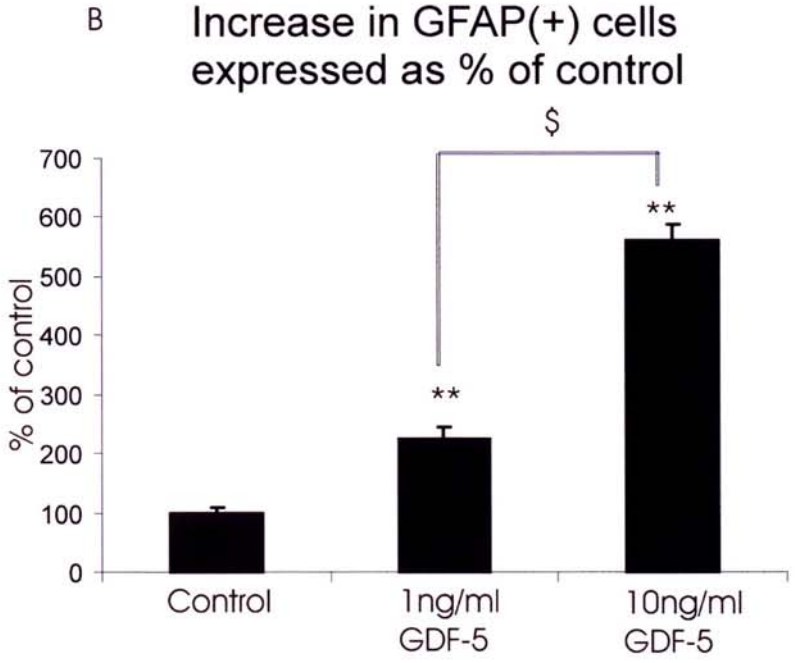

Increase in GFAP(+) cells
expressed as % of control
$
% of control
700
600
500
400
300
200
100
0
**
**
Control
1ng/ml
GDF-5
10ng/ml
GDF-5

Figure 6.5:

RT-PCR analysis of the expression of BMP receptors, BMPR-II, BMPR-Ib, BMPR-Ia, in (A) freshly dissected E14 rat VM tissue and (B) cultures of E14 rat VM grown for 6 DIV. (C) RT-PCR analysis showing the loss of expression of BMPR-Ib in E14 rat VM tissue over 6 days in culture.

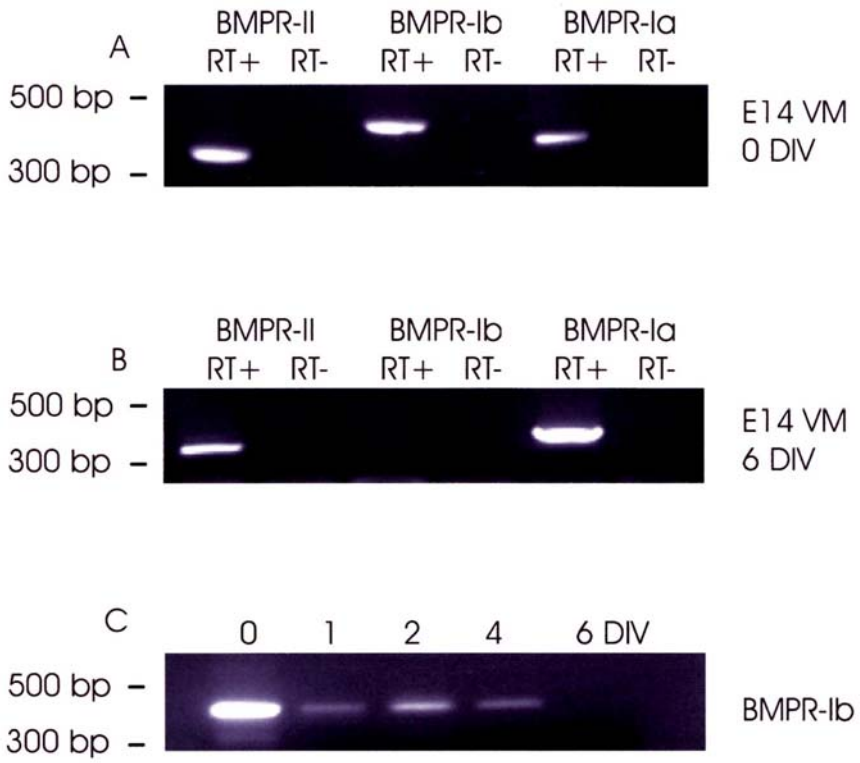


Figure 6.6:

Immunocytochemical analysis of the BMP receptors, (A) BMPR-II, (B) BMPR-Ia and (C) BMPR-Ib in E14 rat VM cultures after 6 DIV. (D) shows a negative control, i.e. a cultures that received no primary antibody.

Scale bar = 50μm.

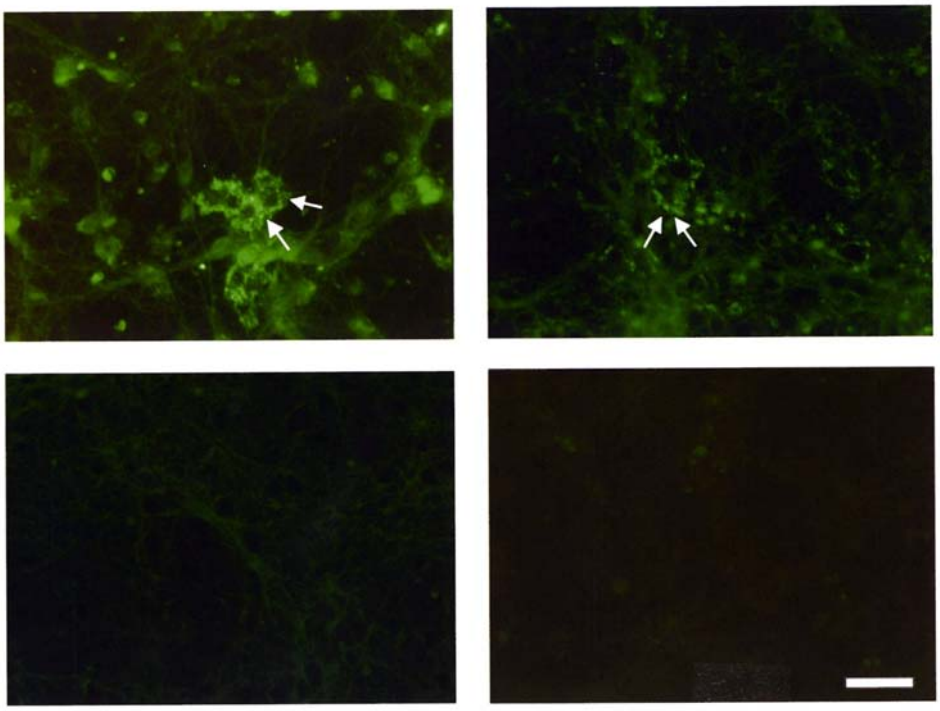
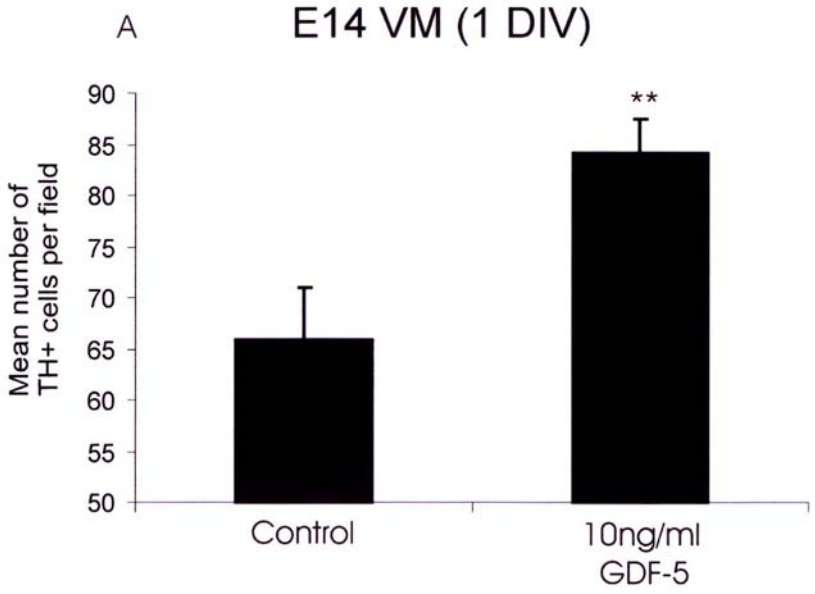


Figure 6.7:

Numbers of dopaminergic neurons per field in E14 rat VM cultures at (A) 1 DIV and (B) 6 DIV following treatment with 10ng/ml GDF-5 for 24 hours. Data represent mean ± SEM taken from two independent experiments.

** $P<0.05$ compared to untreated (control) cultures.


A
E14 VM (1 DIV)
Mean number of TH+ cells per field
90
85
80
75
70
65
60
55
50
**
Control
10ng/ml GDF-5

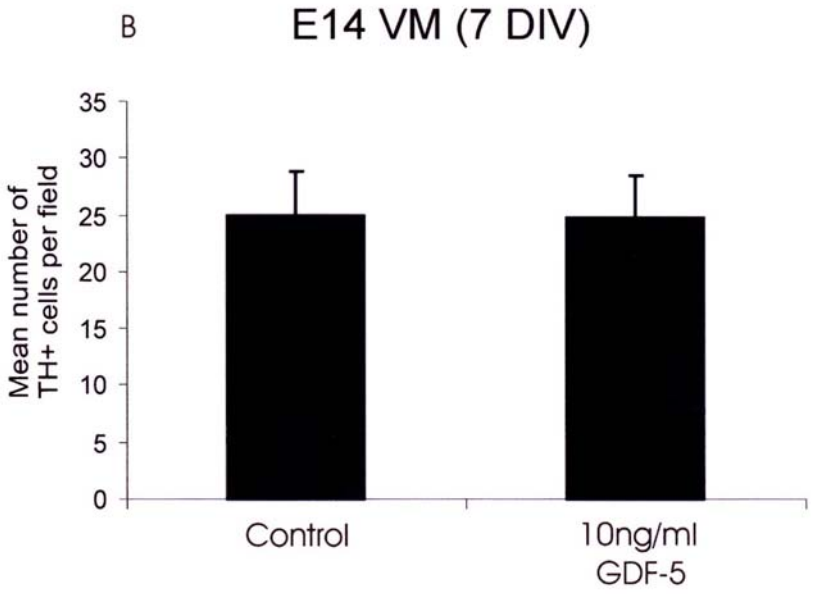

B
E14 VM (7 DIV)
Mean number of TH+ cells per field
35
30
25
20
15
10
5
0
Control
10ng/ml GDF-5

Figure 6.8:

Representative dopaminergic neurons in E14 rat VM culture, immunocytochemically stained for TH after 6 DIV, grown under (A) control conditions, or (B) in medium containing 10ng/ml GDF5.

Scale bar = 20μm.

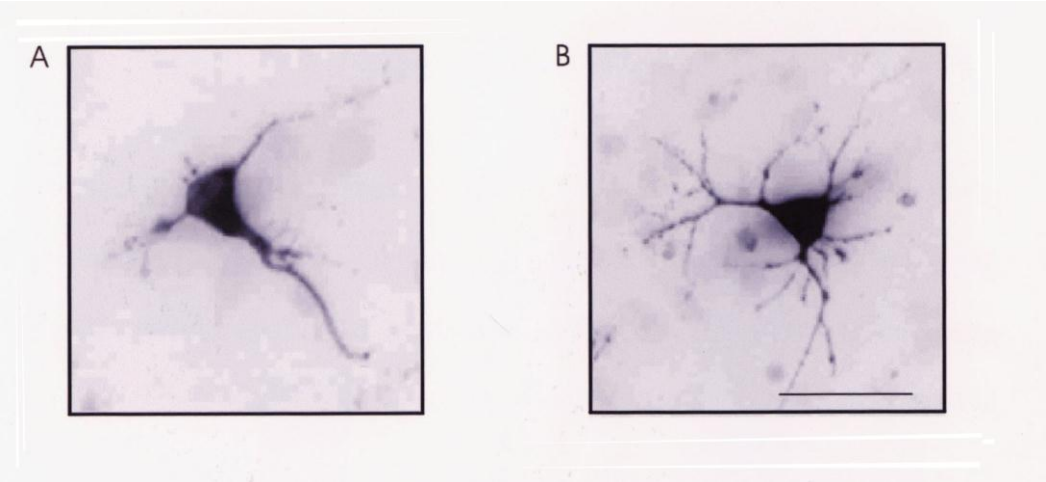
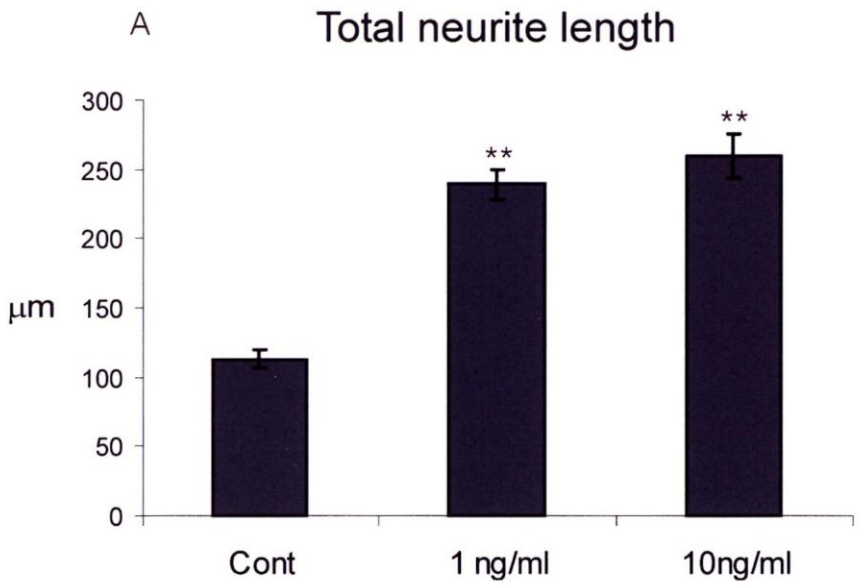
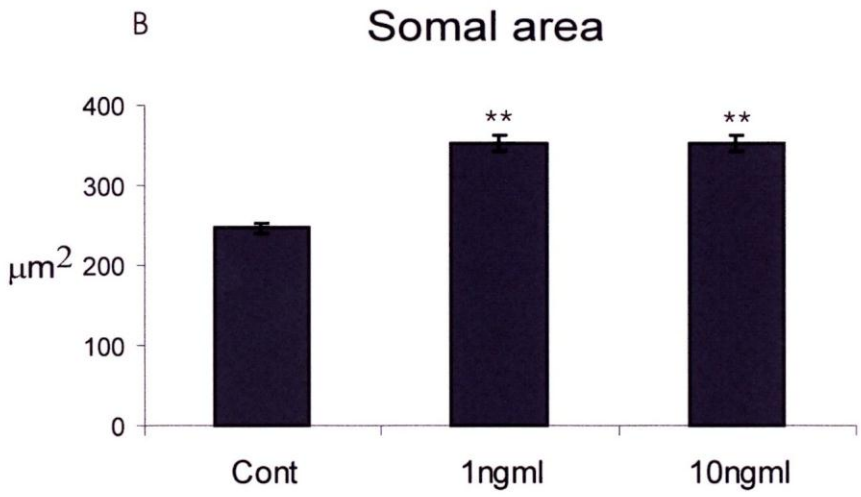


Figure 6.9:

Effects of GDF-5 treatment on (A) total neurite length per dopaminergic neuron, (B) somal area per dopaminergic neuron and (C) number of branches per dopaminergic neuron in E14 rat VM culture after 6 DIV. Data represent mean ± SEM taken from 50 neurons from 3 independent experiments.

** $P<0.001$ compared to untreated (control) cultures.


A
Total neurite length
300
250
200
150
100
50
0
μm
**
**
Cont
1 ng/ml
10ng/ml


B
Somal area
400
300
200
100
0
μm2
**
**
Cont
1ngml
10ngml

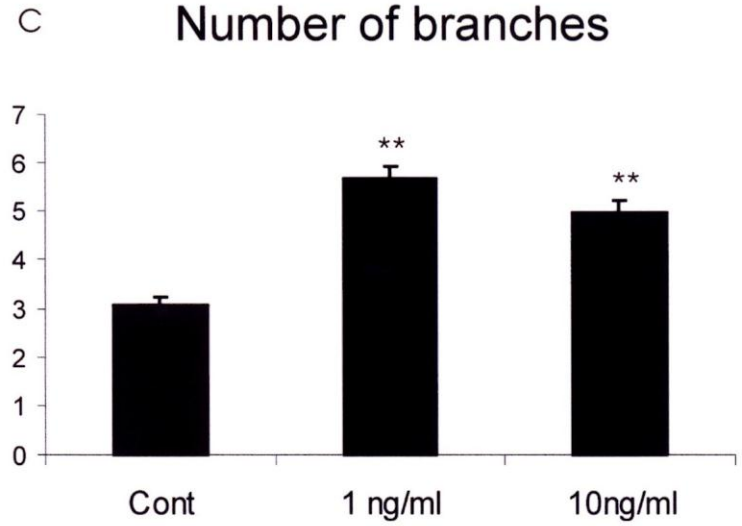
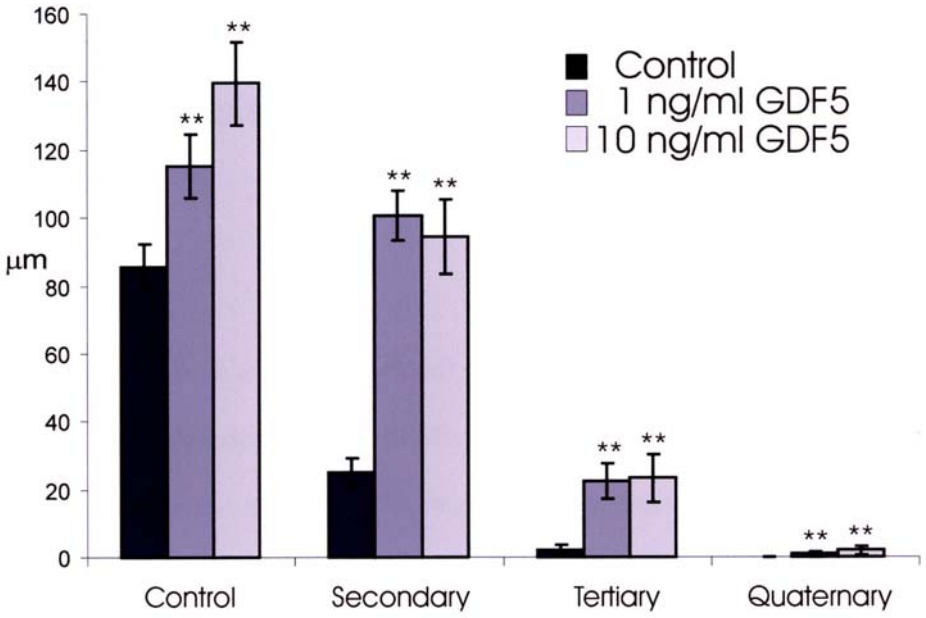

C
Number of branches
7
6
5
4
3
2
1
0
**
**
Cont
1 ng/ml
10ng/ml

Figure 6.10:

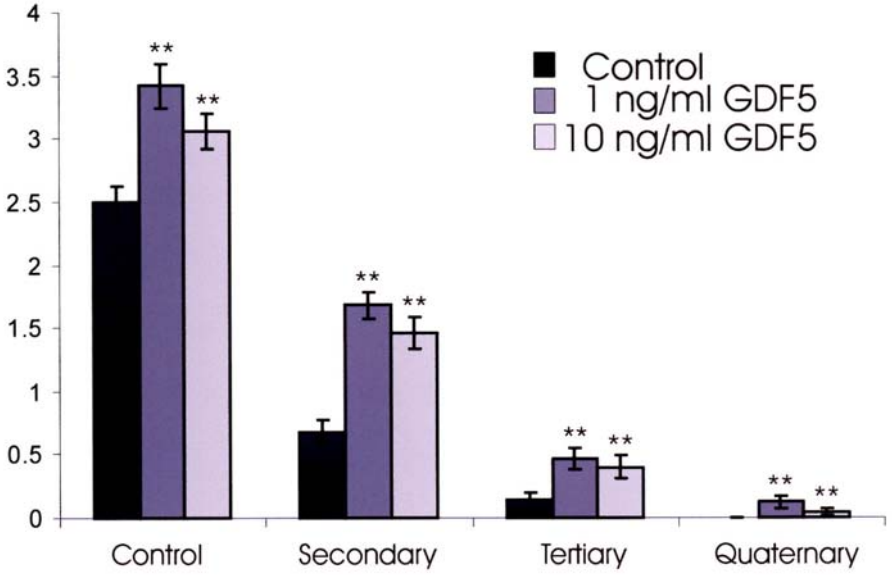
Effects of GDF-5 treatment on (A) neurite length and (B) numbers of branches at each level of the neurite field in dopaminergic neurons in E14 rat VM culture after 6 DIV. Data represent mean ± SEM taken from 50 neurons from 3 independent experiments.

** $P<0.001$ compared to untreated (control) cultures.

A Neurite length at different levels of neurite field



B Branch number at different levels of neurite field



7.0 ANALYSIS OF THE MECHANISM OF GDF-5 INDUCED INCREASES IN TH-POSITIVE CELL NUMBERS AND THE EFFECT OF GDF-5 AND WNT-5a CO-TREATMENT ON TH-POSITIVE CELL NUMBERS IN E14 RAT VM CULTURES

7.1 Aims

- To examine the expression of BMP receptors in the VM of TH-GFP mice.
- To analyse phospho-smad activation by GDF-5 in E14 VM cultures.
- To analyse the mechanism of increase in dopaminergic numbers induced by GDF-5.
- To examine the expression of Ror2 in E14 rat VM.
- To examine the effect on numbers of TH-positive cells when adding GDF-5 and Wnt-5a to cultures of E14 rat VM.

7.2 Introduction

Treatment with GDF-5 has been shown to increase the numbers and improve the morphological development of E14 rat VM dopaminergic neurons (O'Keeffe et al. 2004b; chapter 6). However it is not known whether GDF-5 improves the survival of dopaminergic neurons, or induces a dopaminergic phenotype in uncommitted precursors. Furthermore, little is known about GDF-5's mechanism of action on neuronal cells. For example whether it induces phospho-smad activation. Many studies have examined the effects of adding BMPs to cultures of E14 rat VM (Krieglstein et al. 1995a, 1995b; Jordan et al. 1997; Lingor et al. 1999; Brederlau et al. 2002). However, it is not been shown that embryonic dopaminergic neurons from the VM express BMP receptors, although adult dopaminergic neurons have been shown to express transcripts for BMPR-II (Charytoniuk et al. 2000). All BMPs and GDFs are thought to signal through the serine/threonine kinase receptors (for review see de Caestecker 2004). However recently it has emerged that a tyrosine kinase receptor called Ror2 can act as a receptor for GDF-5 (Dr. P. Tylanowski, personal communication) and Wnt-5a (Oishi et al. 2003). Ror2 (along with Ror1) was cloned from a human neuroblastoma cell line (Masiakowski and Carroll 1992).

As Wnt-5a has previously been shown to increase the numbers of dopaminergic neurons in E14 rat VM cultures (Castelo-Branco et al. 2003), it is possible that GDF-5 and Wnt-5a may have additive or synergistic effects in this system.

7.3 Results

7.3.1 BMP receptors are expressed in VM of TH-GFP mouse

In the TH-GFP mouse, GFP expression is under the control of the TH promoter. As such, cells in the VM that express TH also express GFP. Using FACS sorting, cells can be separated into TH-positive and TH-negative fractions. Real-time PCR analysis using cDNA from the VM of E13.5 TH-GFP mice that was FACS sorted into from TH+ and TH- fractions (a kind gift from Ms. Nina Rawal, Karolinska Institute), showed that BMPR-II and BMPR-Ia were expressed in TH-positive and TH-negative fractions (Figure 7.1A, B respectively). Surprisingly no expression of BMPR-Ib was detected in either TH+ or TH- fractions.

No expression of BMPR-Ib was detected in either the TH-positive or TH-negative fractions in the E13.5 VM. However RT-PCR analysis on RNA extracted from the E14 rat VM showed that that all three BMP receptors are expressed in the rat VM (Chapter 6; Figure 6.5A). A pilot study (data not shown) revealed that BMPR-Ib is expressed in the eight-week old human VM.

7.3.2 GDF-5 stimulates smad activation and nuclear translocation in neurons

Addition of GDF-5 (50ng/ml) for two hs to E14 rat VM cultures which had been growing for 24 hs in serum-free medium, induced nuclear accumulation of phosphoryalted smad-1, -5, -8 (Figure 7.2B). No smad-1, -5, -8 phosphorylation was observed in untreated cultures (Figure 7.2A).

7.3.3 GDF-5 increases the numbers of TH-positive but not TUJ-1-positive, Nurr-1-positive or total cell number in E14 rat VM cultures

Treatment of cultures of E14 rat VM grown for 3 DIV in serum-free conditions with 10ng/ml GDF-5 induced an approximately two-fold increase in the numbers of TH-positive cells when compared to control cultures (Figure 7.3A, B, C; $P<0.01$). No difference was observed between neuronal numbers in control cultures and GDF-5 treated cultures (Figure 7.4A, B, C). No difference was also observed between the total numbers of cells in control cultures and GDF-5 treated cultures (Figure 7.5A, B, C). When the data was expressed as percentages of total cells, GDF-5 increased the percentage of neurons (Figure 7.6A; $P<0.01$) and the percentage of cells that expressed TH (Figure 7.6B; $P<0.01$). No difference was observed between the numbers of Nurr-1 positive cells in control cultures and GDF-5 treated cultures (Figure 7.7A, B, C), whereas the percentage of Nurr-1 positive cells that expressed TH in GDF-5 treated cultures was significantly increased (Figure 7.7D; $P<0.05$). No difference was observed between the numbers of pyknotic nuclei in control cultures and GDF-5 treated cultures (Figure 7.8).

7.3.4 GDF-5 and Wnt-5a do not have additive effects to increase the numbers of dopaminergic neurons in cultures of E14 rat VM

RT-PCR analysis showed that Ror2 was strongly expressed in RNA extracted from the E14 rat VM (Figure 7.9A). The presence of this novel receptor for both GDF-5 and Wnt-5a may allow these factors to increase the numbers of TH-positive neurons in cultures of E14 rat VM to a greater extent than either factor alone. To examine this further, cultures of E14 rat VM were treated with Wnt-5a, GDF-5 or a combination of both. As observed previously, GDF-5 treatment of E14 rat VM cultures for 3 DIV resulted in an approximately three-fold increase in the percentage of neurons expressing TH (Figure 7.9B; $P<0.01$) and approximately a two-fold increase in the percentage of total cells that expressed TH (Figure 7.9C; $P<0.01$). Wnt-5a treatment induced similar

increases in the percentage of neurons that expressed TH (Figure 7.9B; $P<0.01$) and in the percentage of total cells that expressed TH (Figure 7.9C; $P<0.01$). No significant synergistic or additive effects were seen in either parameter following GDF-5 and Wnt-5a co-treatment (Figure 7.9B, C). A control for Wnt-5a involved adding to the cultures the buffer used in the extraction of Wnt-5a. No significant increase in the percentage of neurons expressing TH (Figure 7.10B) or in the percentage of total numbers of cells expressing TH was found on addition of the extraction buffer (Figure 7.9C).

7.4 Discussion

7.4.1 Species differences exist between mouse and rat in terms of BMP receptor expression

Previously it has been shown that GDF-5 signals most efficiently through BMPR-II and BMPR-Ib, but not through BMPR-Ia in the rat osteo-progenitor like cell line ROB-C26 (Nisitoh et al. 1996). The present finding that BMPR-Ib is not expressed in the E13 mouse VM is in agreement with previous reports showing that BMPR-Ib is not expressed in the developing mouse VM (Dewulf et al. 1995; Zhang et al. 1998), and that at E15 the expression of BMPR-Ib in the mouse brain was restricted to the frontal region of the VZ and the olafactory neuroepithelium (Zhang et al. 1998). The present finding that BMPR-II and BMPR-Ia are expressed on TH-positive neurons in the mouse VM suggests that these neurons are subject to BMP signalling at this stage of their development. However a previous study has shown that BMPR-II, BMPR-Ia and BMPR-Ib are all expressed in the rat VM of equivalent development age (O'Keeffe et al. 2004b; see Chapter 6), suggesting that fundamental species differences exist between mouse and rat in terms of the expression of BMP receptors.

These findings raise the important issue about which experimental system most closely represents the human VM at an equivalent stage of development. As GDF-5 improves the survival of E14 rat VM dopaminergic neurons after grafting in a rat 6-OHDA model of PD (Sullivan et al. 1998) it has become a candidate neurotrophin for the use in the treatment of PD. This is supported by the observation that BMPR-Ib is expressed in the rat VM at E14 (O'Keeffe et al. 2004b; see Chapter 6). However BMPR-Ib is not expressed whereas in the mouse E13 VM (present study; Zhang et al. 1998), which suggests that GDF-5 may not be a neurotrophic factor for mouse VM dopaminergic neurons. If the human VM does not express BMPR-Ib it is unlikely that

GDF-5 could be applied to the treatment of PD in humans. However, a pilot study carried out as a follow-up to the present set of experiments showed that BMPR-II, BMPR-Ia *and* BMPR-Ib are all expressed in the eight-week old human VM (O'Keeffe et al. *unpublished observations*). These findings show that care should be taken when interpreting the findings of studies examining the effects of BMPs on mouse VM dopaminergic neurons. Furthermore they suggest that the E14 rat VM more closely mirrors the human VM of similar developmental status and should be used in experimental settings to examine the potential neurotrophic effect of BMPs and GDFs on dopaminergic neurons. It also suggests that GDF-5 may be useful in the clinical setting for the treatment as the appropriate receptors exist for it to have a neurotrophic effect.

7.4.2 GDF-5 phosphorylates smad protein in cultures of E14 rat VM

Binding of BMPs and GDFs to their appropriate type II receptor results in phosphorylation of type I receptors. Through a series of phosphorylations and protein interactions, these activate smad effector proteins which move into the nucleus and affect transcription (for review see ten Dijke, P. et al. 2000). Previously it has been shown that BMP-5, -6 and BMP-7, but not GDF-5, activated and induced the nuclear translocation of phosphorylated smad proteins when applied to cultures of E14 rat VM after 7DIV (Brederlau et al. 2002). However, the present findings have shown that with increasing time in culture, BMPR-Ib expression is lost, whereas BMPR-II and BMPR-Ia remain strongly expressed until at least 7DIV (O'Keeffe et al. 2004b; Chapter 6). In the present set of experiments, these findings have been expanded to show that when GDF-5 was applied to cultures of E14 rat VM at a time when BMPR-Ib remains to be expressed (1DIV), smad activation and nuclear translocation occured in a subset of TUJ-1-positive cells. GDF-5 also has been reported to activate p38 MAPK and Erk signal transduction

pathways in a mouse chondrogenic cell line, ADTC5 (Nakamura et al. 1999). A recent study has also shown that BMP-5 activates smad-phosphorylation and p38 MAPK signal transduction during chick limb development (Zuzarte-Luís et al. 2004). Interestingly, it was found that certain genes were induced by BMP-5 in a p38 MAPK-dependant manner, while other genes were induced in a p-38 MAPK independent manner (Zuzarte-Luís et al. 2004). Although the present set of experiments have shown that GDF-5 stimulates smad activation in neuronal cells, it cannot be ruled out that the effects of GDF-5 on the numbers of TH-positive cells and on the morphological development of these cells *in vitro* (O'Keeffe et al. 2004b; Chapter 6) are due at least in part to a smad-independent pathway.

7.4.3 GDF-5 increases the proportion of neurons that acquire a TH phenotype

Although previous experiments had established that GDF-5 increases the numbers of TH-positive neurons in cultures of E14 rat VM (Krieglstein et al. 1995b; O'Keeffe et al. 2004b; see Chapter 6) it was not know whether it also affected other neurons in these cultures. A single dose of 10ng/ml GDF-5 added to cultures of E14 rat VM at the time of plating resulted in a two-fold increase in the numbers of TH-positive cells after 3 DIV but no differences were observed in the numbers of TUJ-1-positive cells or in the total numbers of cells in these cultures. As the proportion of TH-positive neurons of the total TUJ-1-positive neurons was increased, this data shows that GDF-5 selectively affects VM dopamine neurons and promotes the acquisition of a dopaminergic phenotype.

The effects of GDF-5 reported here are similar to those previously reported for Wnt-5a (Castelo-Branco et al. 2003). Wnt-5a is a member of the Wnt family of secreted glycoproteins that is expressed in the developing VM (Parr et al. 1993; Castelo-Branco et al. 2003). Wnt-5a did not affect the numbers of neurons in cultures of E14 rat VM after 3 DIV, but resulted in an approximately two-fold increase in the numbers of TH-

positive neurons and in the proportion of TH-positive neurons of the total TUJ-1-positive neurons (Castelo-Branco et al. 2003). Wnt-5a also increased the proportion of nurr1-positive cells that were TH-positive. The present findings suggest that GDF-5 acts in a similar manner to Wnt-5a in cultures of E14 rat VM.

7.4.4 GDF-5 and Wnt-5a act on a similar dopaminergic precursor cells

In agreement with previous findings showing that Ror2 is expressed in the mouse VM at E9.5 (Takeuchi et al. 2000), the present study found the expression of Ror2 in the rat VM at E14. As Ror2 can act as a receptor for both GDF-5 (Dr. P. Tylanowski personal communication) and Wnt-5a (Oishi et al. 2003), it is not surprising that is expressed in the E14 rat VM when both GDF-5 (O'Keeffe et al. 2004a; Chapter 5) and Wnt-5a (Castelo-Branco et al. 2003) are expressed at high levels. The expression of Ror2 has been shown to peak at around E16 in the mouse brain and to decrease with increasing age reaching its lowest level in the adult (Paganoni and Ferreira 2003). A previous report has shown that Ror2 is expressed in the growth cones of immature cultures of E16 mouse hippocampal neurons (Paganoni and Ferreira 2003), possibly indicating a role in neurite outgrowth. Since it is also expressed on immature dopaminergic neurons, it is tempting to speculate that the effects of GDF-5 on the morphological development of dopaminergic neurons (O'Keeffe et al. 2004b; Chapter 6) may be (at least in part) mediated by Ror2. Ror2 has also been shown to be expressed in cortical astrocytes (Paganoni et al. 2004). Thus it is possible that the increase in astrocyte number induced by GDF-5 in cultures of E14 rat VM may be mediated by Ror2. However, it remains to be determined whether Ror2 is expressed in astrocytes in these cultures. The establishment of the precise role(s) (if any), that Ror2 plays in GDF-5 signalling will require further studies.

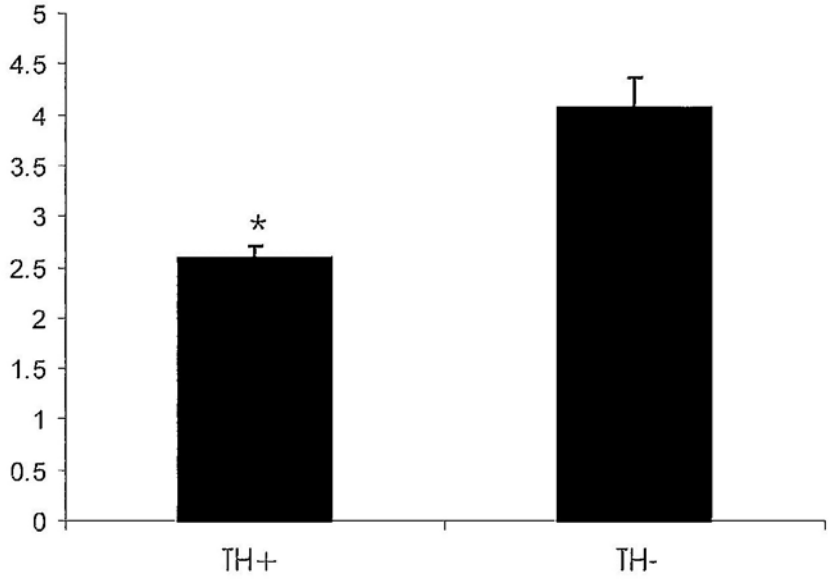
As previously mentioned, Wnt-5a is expressed in the rat VM at E13.5. Wnt-5a treatment increases the numbers of dopaminergic neurons in cultures of E14 rat VM by increasing the proportion of Nurr1-positive precursors that acquired a dopaminergic phenotype (Castelo-Branco et al. 2003). The present study found that GDF-5 acts in a similar manner; increasing numbers of dopaminergic neurons in cultures of E14 rat VM by increasing the proportion of nurr1-positive precursors that acquire a dopaminergic phenotype. These findings suggest that GDF-5 and Wnt-5a could potentially have synergistic effects on dopaminergic neurons in cultures of E14 rat VM. Transcriptional regulation of certain Wnt target genes occurs through nuclear translocation of a transcriptional complex containing β-catenin and a member of the Tcf family (for review see Polakis 2000), while BMP signalling requires nuclear translocation of activated smad proteins as previously discussed (for review see de Caestecker et al. 2004). Synergy between TGF-β and Wnt signalling has also been shown to regulate developmental events in a variety of tissues (Cadigan and Nusse 1997; Whitman 1998). A previous report has shown that BMP and Wnt signalling cooperate to regulate the expression of the transcription factor *Emx2* in the dorsal mouse telencephalon (Theil et al. 2002). The present set of experiments found that the effect of adding both GDF-5 and Wnt-5a to cultures of E14 rat VM was the same as adding GDF-5 or Wnt-5a alone. This suggests that these factors act on the same population of precursor cells in these cultures and do not act in a synergistic fashion to increase dopaminergic neuron number in cultures of the E14 rat VM.

Figure 7.1:

Real-time PCR analysis of the expression of (A) BMPR-II and (B) BMPR-Ia in FACS-sorteed TH-positive and TH-fractions of the VM of the E13 TH-GFP mouse. Data represent mean ± SEM from 6 repetitions of the PCR per sample.

* $P<0.05$ compared to TH- fraction.

A Real-time PCR analysis of BMPR-II expression in VM of E13 TH-GFP mouse



B Real-time PCR analysis of BMPR-Ia expression in VM of E13 TH-GFP mouse

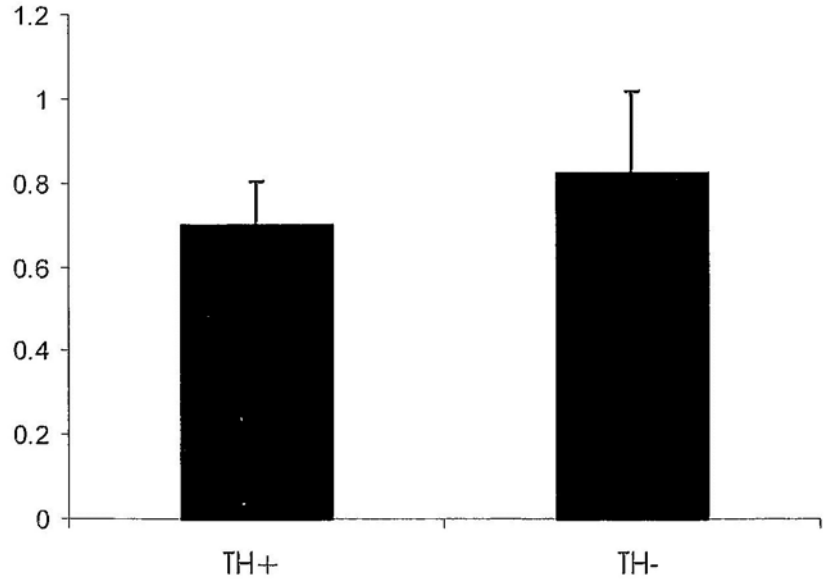


Figure 7.2:

Photomicrographs showing representative cultures of E14 rat VM grown for 1 DIV. GDF-5 was added to the culture medium after 24 hours and cells were allowed to survive for a further 2 hours. Cultures were fixed and immunocytochemically stained for TUJ (red) and phosphorylated smad-1, -5, -8 (green). No smad activation was seen in (A) control cultures, whereas in (B) GDF-5 treated cultures, nuclear accumalation of phosphorylated smad protein was seen in a subset of neurons.

Scale bar = 25μm

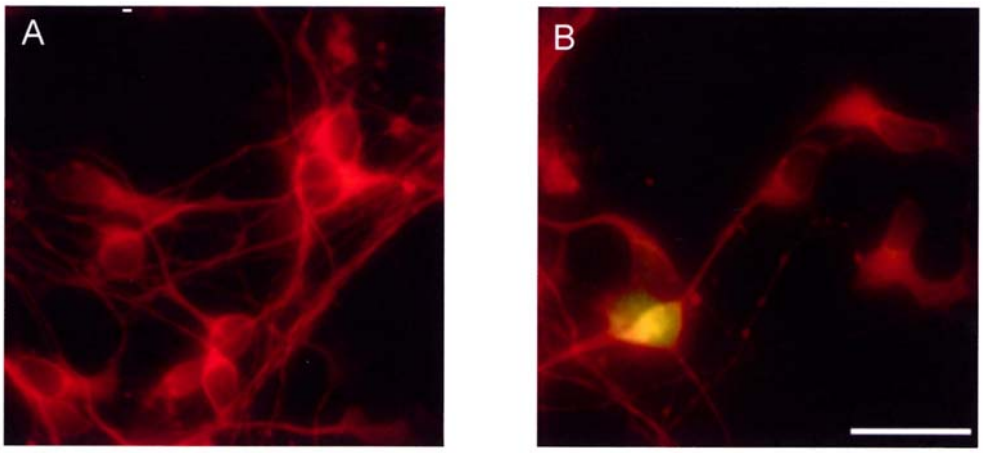
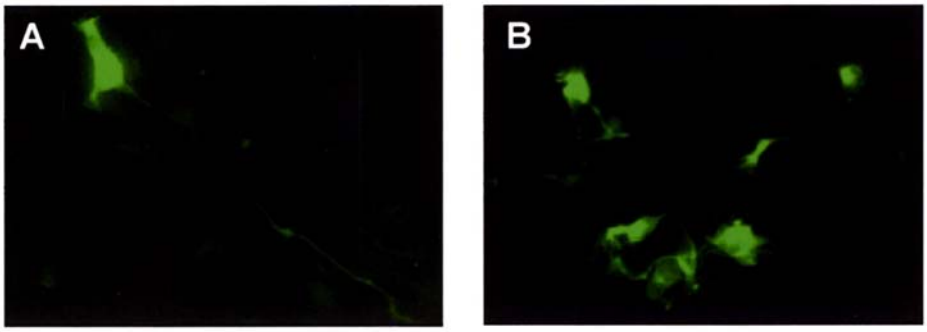


Figure 7.3:

Photomicrographs showing representative cultures of E14 rat VM grown for 3 DIV, immunocytochemically stained for TH. (A) shows a control culture, (B) shows a culture that received 10ng/ml GDF-5 at time of plating. (C) Graphical representation of mean numbers of TH-positive cells per field in control cultures and cultures treated with 10ng/ml GDF-5. Data represent mean ± SEM taken from three independent experiments.

**$P<0.01$ when compared to control cultures.



C Mean number of TH-positive cells per field

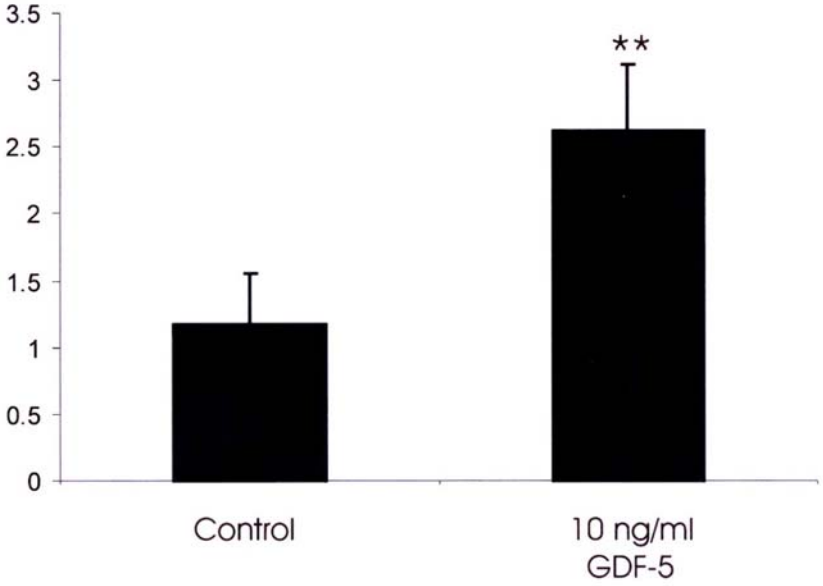


Figure 7.4:

Photomicrographs showing representative cultures of E14 rat VM grown for 3 DIV, immunocytochemically stained for TUJ. (A) shows a control culture, (B) shows a culture that received 10ng/ml GDF-5 at time of plating. (C) Graphical representation of mean numbers of TUJ-positive cells per field in control cultures and cultures treated with 10ng/ml GDF-5. Data represent mean ± SEM taken from three independent experiments.

N.S. no significant difference.

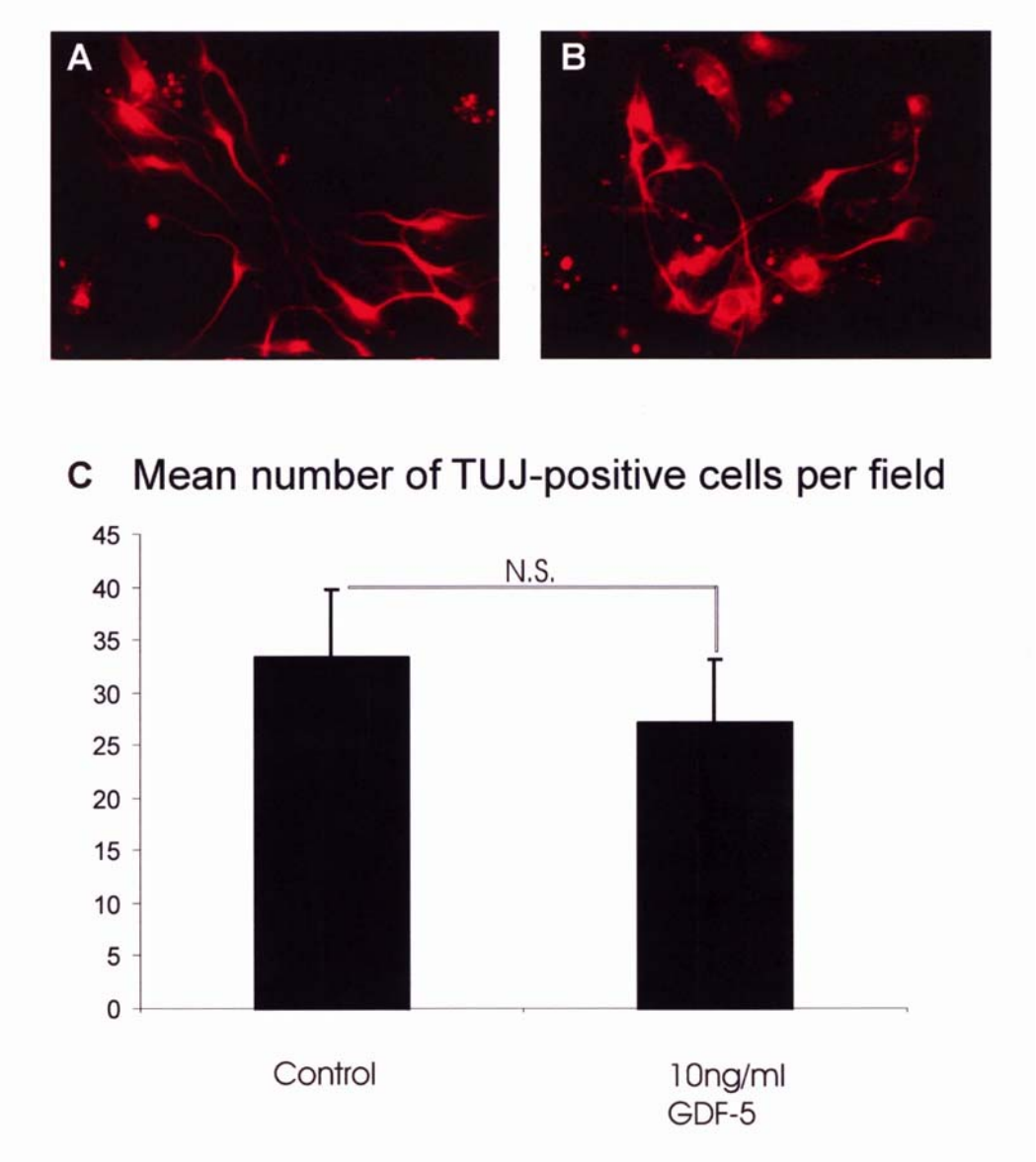


Figure 7.5:

Photomicrographs showing representative cultures of E14 rat VM grown for 3 DIV, immunocytochemically stained for DAPI. (A) shows a control culture, (B) shows a culture that received 10ng/ml GDF-5 at time of plating. (C) Graphical representation of mean numbers of DAPI-positive cells per field in control cultures and cultures treated with 10ng/ml GDF-5. Data represent mean ± SEM taken from three independent experiments.

N.S. no significant difference.

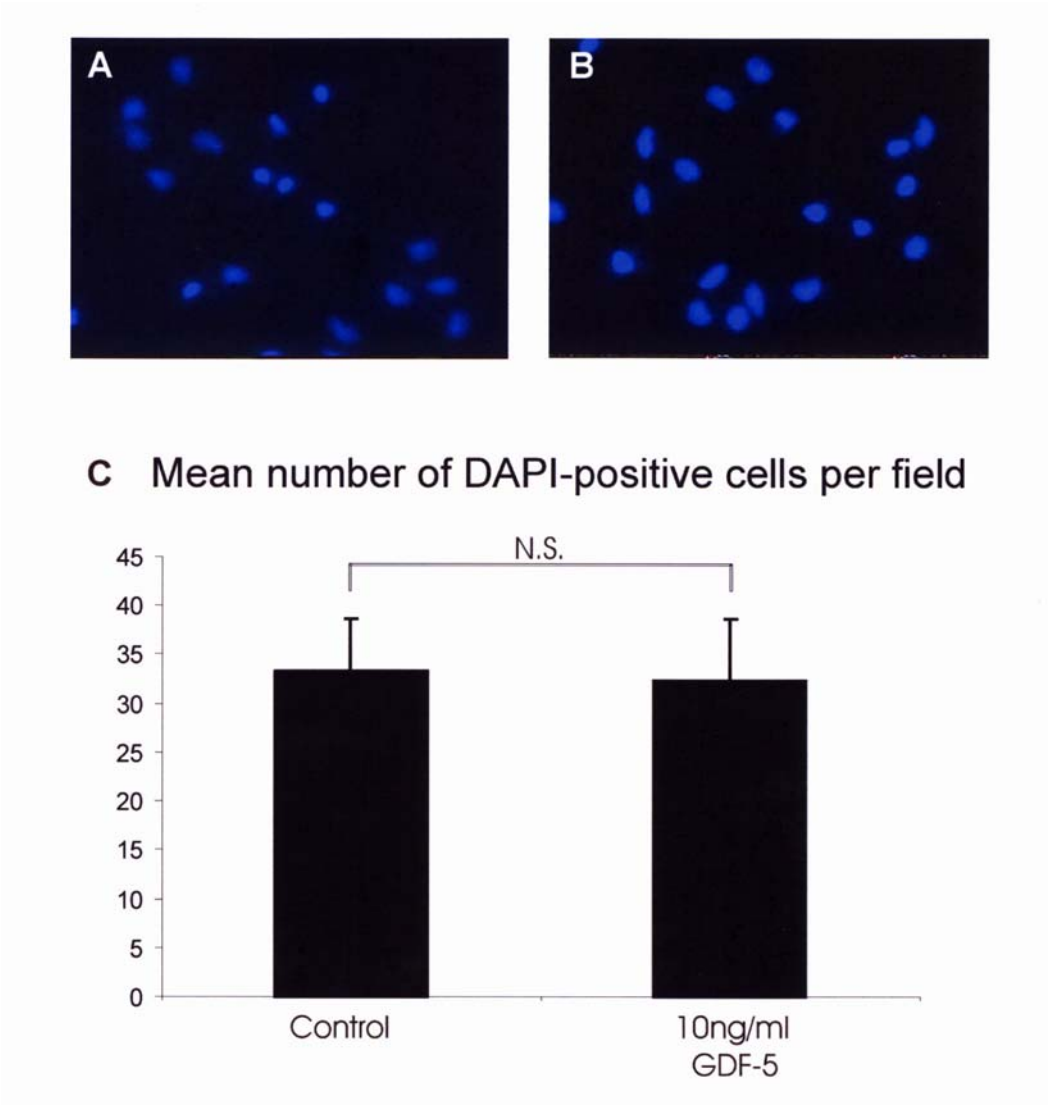


Figure 7.6:

Graphical representation of (A) the percentage of neurons and (B) the percentage of total cells that were TH-positive in cultures of E14 rat VM grown for 3 DIV, immunocytochemically double-stained for (A) TH/TUJ or (B) TH/DAPI. Data represent mean ± SEM taken from three independent experiments.

**P<0.01 compared to control cultures.

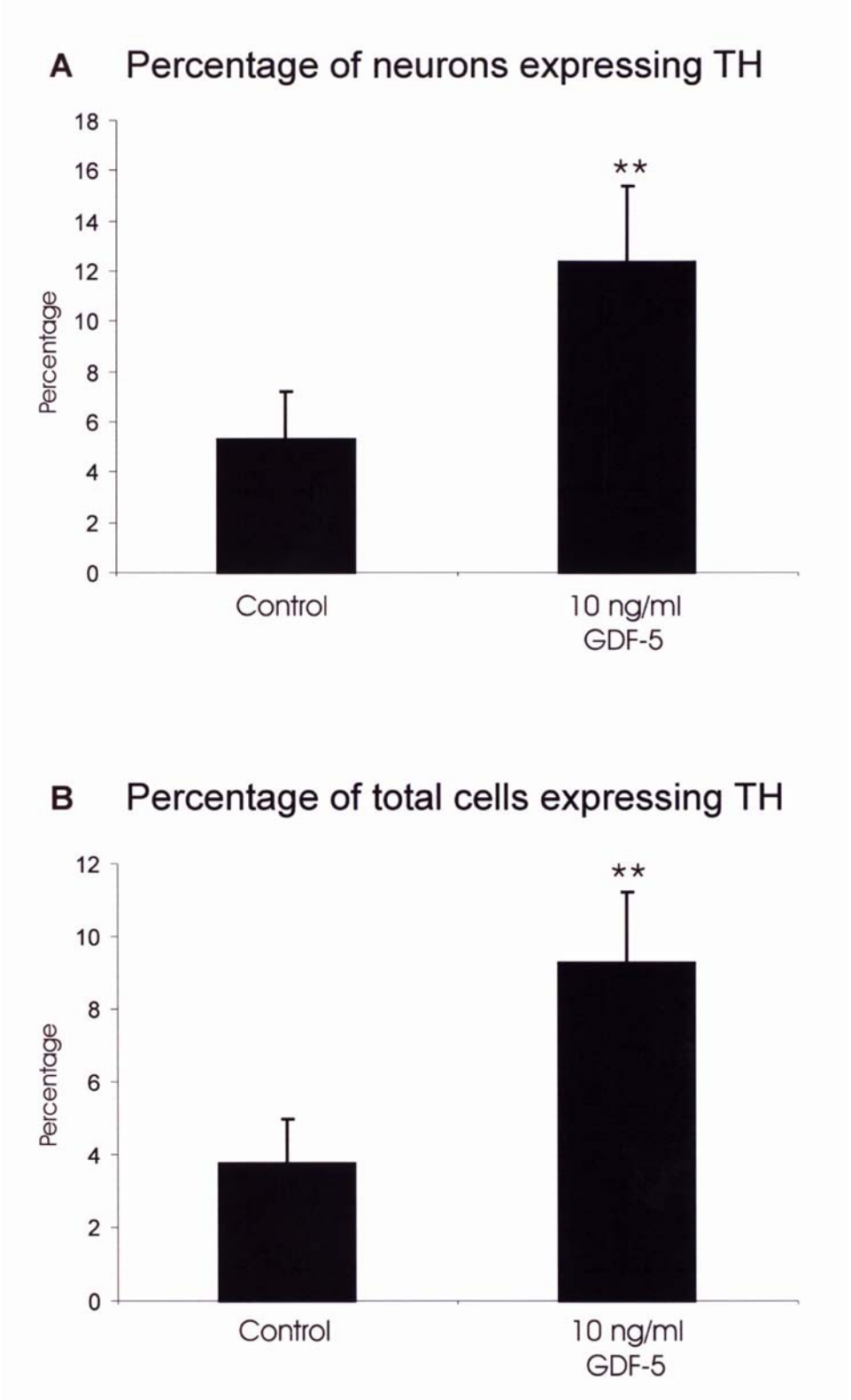
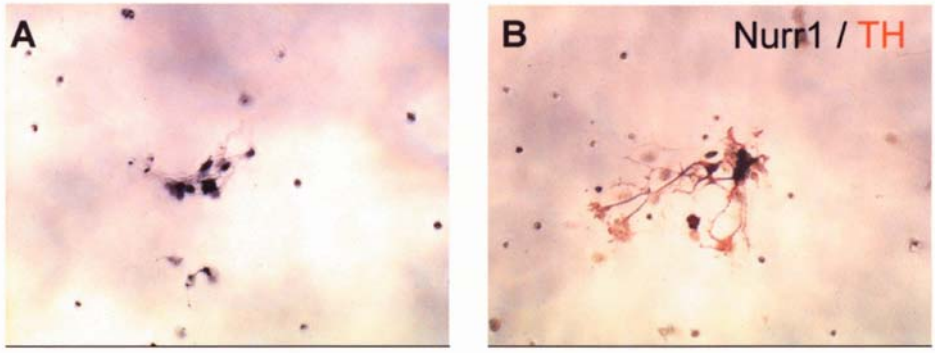

A
Percentage of neurons expressing TH
Percentage
18
16
14
12
10
8
6
4
2
0
**
Control
10 ng/ml
GDF-5
B
Percentage of total cells expressing TH
Percentage
12
10
8
6
4
2
0
**
Control
10 ng/ml
GDF-5

Figure 7.7:

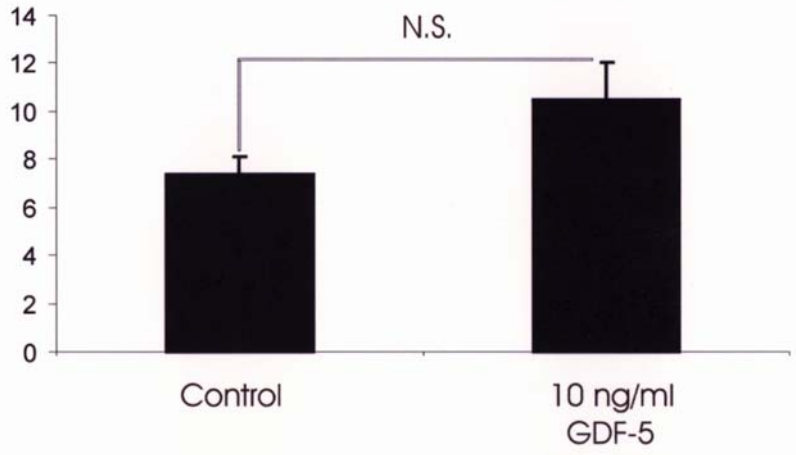
Photomicrographs showing representative cultures of E14 rat VM grown for 3 DIV, immunocytochemically stained for Nurr1 (black) and TH (brown). (A) shows a control culture, (B) shows a culture that received 10ng/ml GDF-5 at time of plating. (C) Graphical representation of mean numbers of nurr1-positive cells per field in control cultures and cultures treated with 10ng/ml GDF-5. (D) Graphical representation showing the percentage of nurr1-positive cells that also expressed TH in control cultures and cultures treated with 10ng/ml GDF-5. Data represent mean ± SEM taken from three independent experiments.

*$P<0.05$ when compared to control cultures.

N.S. no significant difference.



C Mean number of Nurr1-positive cells per field



D Percentage of Nurr1 cells expressing TH

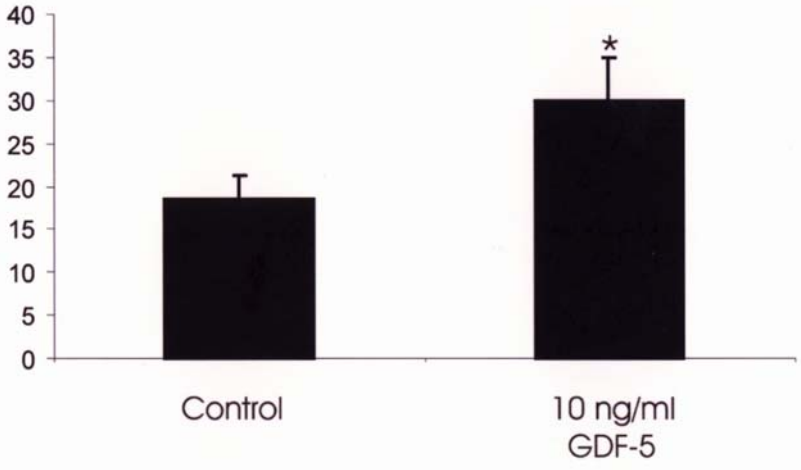


Figure 7.8:

Graphical representation of mean numbers of apoptotic nuclei per field in control cultures and cultures treated with 10ng/ml GDF-5 as detected by DAPI staining. N.S. no significant difference

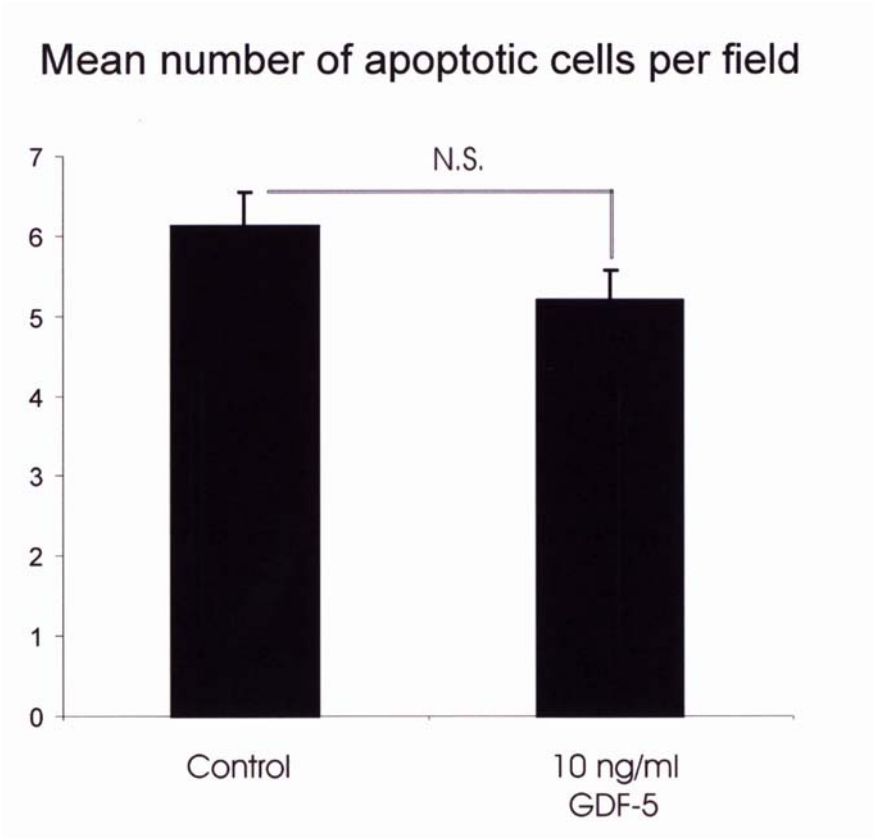


Figure 7.9:

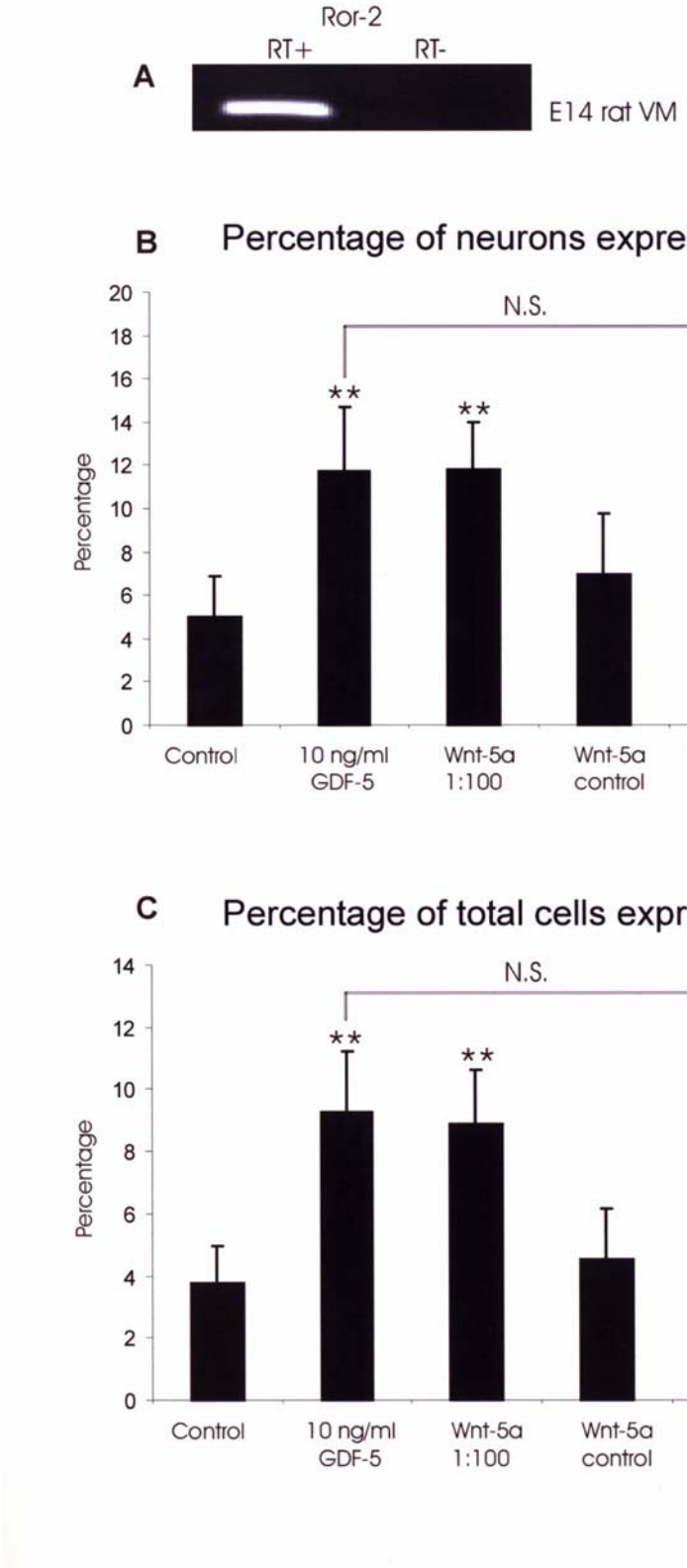
(A) RT-PCR analysis of the expression of the Ror2 receptor in freshly dissected E14 rat VM tissue. Graphical representation showing (B) the percentage of neurons and (C) the percentage of total cells that were TH-positive in cultures of E14 rat VM grown for 3 DIV, then immunocytochemically double-stained for (B) TH/TUJ or (C) TH/DAPI that were treated with GDF-5, Wnt-5a or a combination of both.

Data represent mean ± SEM taken from two independent experiments

**P<0.01 compared to control cultures

*P<0.05 compared to control cultures

N.S. no significant difference


A
Ror-2
RT+
RT-
E14 rat VM
B
Percentage of neurons expressing TH
Percentage
N.S.
**
Control
10 ng/ml GDF-5
Wnt-5a 1:100
Wnt-5a control
10 ng/ml GDF-5 + Wnt-5a 1:100
10 ng/ml GDF-5 + Wnt-5a control
C
Percentage of total cells expressing TH
Percentage
N.S.
**
*
Control
10 ng/ml GDF-5
Wnt-5a 1:100
Wnt-5a control
10 ng/ml GDF-5 + Wnt-5a 1:100
10 ng/ml GDF-5 + Wnt-5a control

8.0 AGE-RELATED DIFFERENCES IN THE ABILITY OF EXPANDED VENTRAL MESENCEPHALIC PROGENITORS TO GENERATE DISTINCT CELL TYPES WHEN INDUCED TO DIFFERENTIATE

8.1 Aims

- To compare the volume and diameter of neurospheres derived from E12, E13 and E14 rat VM
- To examine whether the age of donor tissue influences ability to differentiate into distinct cell phenotypes
- To examine dopaminergic neuronal differentiation from expanded NPC from E12, E13 and E14 rat VM.

8.2 Introduction

NSCs exist in the developing and adult brain which give rise to progenitor cells capable of differentiating into the major cell types of the nervous system, neurons, astrocytes or oligodendrocytes (for review see Gage, 2000). These neural stem cells can be isolated from the embryonic mouse (Reynolds et al. 1992), rat (Svendsen et al. 1995) and human (Carpenter et al. 1999) CNS. NSCs can proliferate in culture in response to EGF and FGF-2, generating structures called "neurospheres", which are free-floating spherical aggregates. Cells in these neurospheres express the intermediate filament protein nestin, which is used as a marker of multipotential stem cells (Frederiksen *et al.* 1988). Cells derived from these neurospheres can subsequently give rise to neurons, astrocytes and oligodendrocytes (for review see Gage 2000). Thus these cells can be used to study lineage specification from NSCs and have the potential to generate cells for transplantation approaches to the treatment of neurodegenerative diseases (for reviews see Svendsen and Smith 1999; Caldwell 2001; Rossi and Cattaneo 2002).

Transplantation of embryonic dopaminergic neurons to the damaged brain is a potential treatment for PD, however one of the main limiting factors to this approach is the poor survival of these neurons after transplantation (for review see Brundin et al 2000). Due to the large numbers of dopaminergic cells required for transplantation, alternative sources of these cells are being sought. Expanded precursors from the developing mouse (Studer et al. 1998), rodent (Ostenfeld et al. 2002) and human (Carpenter et al. 1999; Ostenfeld et al. 2002) midbrain have been shown to have the potential to differentiate into TH-expressing dopaminergic neurons *in vitro* and following transplantation to the CNS (Studer et al. 1998). As such, they may be of use to generate an alternative supply of dopaminergic neurons for transplantation approaches to Parkinson's disease.

Although many groups have expanded cells from the embryonic rodent VM, the

age of donor tissue used in these experiments varies. E12 (Studer et al. 1998), E14 (Ostenfeld et al. 2002) and E18 (Svendsen et al. 1995) VM have all been used to generate neurospheres. In the rat VM, dopaminergic neurons proliferate between E11 and E15, undergoing their final mitotic divisions on E14-15 (Altman and Bayer 1981; Bayer et al. 1995). Thus dopaminergic neuronal precursor cells in an undifferentiated state would be expected to be present in the rat VM from E11 to E14, after which point they may become post-mitotic. In the present set of experiments, the yields of various cell types generated from neurospheres derived from E12, E13 and E14 rat VM were compared, as was the ability of these neurospheres to give rise to dopaminergic neurons. The results show that the percentage of each cell type generated in these cultures varies with the age of the donor tissue. The ability of the neurospheres to generate dopmainergic neurons was also found to be age dependent. The size of neurospheres derived from each of the three ages was also examined.

8.3 Results

8.3.1 Neurosphere characteristics during expansion phase

Dissociated cells from E12, E13 and E14 VM, seeded into expansion medium, were seen to form small clusters after 1 DIV, with these clusters proliferating into recognizable neurospheres after 2 DIV. The diameter of the neurospheres from each age, approximately doubled every 2 DIV (Figure 8.1-8.3; Appendix III). By 6 DIV for E13 and as early as 4 DIV for E14, some of the neurospheres in these cultures stuck to the bottom of the flask, with "islands" of cells forming around them (Figure 8.3B, C). These attached spheres continued to increase in size. Interestingly, very few of the E12 neurospheres ever stuck to the bottom of the flask, the majority of them remained free-floating (Figure 8.1A, 8.2A, 8.3A). After 6 DIV of expansion, all the spheres were nestin positive, indicating their multipotentiality.

8.3.2 Comparison of sphere diameter and volume during expansion phase

During the expansion phase, neurospheres from E12 and E13 cultures were very similar in size, with no significant difference in the diameter of these neurospheres at 2, 4 or 6 DIV (Figure 8.1D, 8.2D, 8.3D respectively). However, the diameter of neurospheres from E14 cultures was significantly smaller than that of neurospheres from E12 and E13 cultures at 2, 4, and 6 DIV (Figure 8.1D, 8.2D, 8.3D respectively; $P<0.01$). The volumes of neurospheres from E12 and E13 cultures were not significantly different at 2, 4 or 6 DIV (Figure 8.1E, 8.2E, 8.3E respectively). The volume of neurospheres from E14 cultures was significantly smaller than that of neurospheres from E12 and E13 cultures at 2, 4, and 6 DIV (Figure 8.1E, 8.2E, 8.3E respectively; $P<0.01$).

8.3.3 Differentiation of expanded VM neurospheres

After 6 DIV expansion, neurospheres were dissociated to single cells and plated onto poly-D-lysine coated wells and allowed to differentiate after for a further 6 DIV, in the presence of 1% FCS, without EGF and FGF-2. At 24 h after plating, the cells derived from neurospheres of all ages had developed processes, but lacked the morphological characteristics of mature neuronal cell types. After 6 DIV, cultures were fixed and stained for TUJ-1, MAP-2ab, GFAP, MBP and nestin. Cells immunoreactive for all markers were found in cultures from neurospheres of all ages, but the percentage of the different cell type generated varied with the age of the culture (Figure 8.4 – 8.9).

8.3.4 Neuronal differentiation

E12 cultures contained the highest percentage of neurons (TUJ-1-positive cells; 19.3 ± 0.6%, MAP-2ab-positive cells; 17.2 ± 0.6%; Figure 8.4A, Figure 8.5A respectively), with E13 (TUJ-1-positive cells; 12.8 ± 1.9%, MAP-2ab-positive cells; 11.9 ± 0.6%; Figure 8.4B, Figure 8.5B respectively) and E14 (TUJ-1-positive cells; 6.9 ± 1.2%, MAP-2ab; 3.5 ± 0.4%; Figure 8.4C, Figure 8.5C respectively) having significantly fewer neurons (Figure 8.4D, Figure 8.5D respectively; $P < 0.01$). The percentage neurons generated in E14 cultures was significantly lower than that in E13 cultures (Figure 8.4D, Figure 8.5D respectively; $P < 0.01$).

8.3.5 Astro/Oligodendroglial differentiation

E12 cultures contained the lowest percentage of GFAP-positive cells (9.4 ± 0.6%; Figure 8.6A), with E13 (48.6 ± 3.8%; Figure 8.6B) and E14 (44.0 ± 2.0%; Figure 8.6C) cultures having significantly more GFAP-positive cells (Figure 8.6D; $P<0.01$). There was no significant difference between the percentage of GFAP-positive cells in E13 and E14 cultures (Figure 8.6D).

E12 cultures contained the lowest percentage of MBP-positive cells (3.7 ± 2.0%; Figure 8.7A), with E13 (5.5 ± 0.6%; Figure 8.7B) and E14 (13.5 ± 1.4%; Figure 8.7C) cultures having significantly more MBP-positive cells (Figure 8.7D; $P < 0.01$). There was a significant difference between the percentage of MBP-positive cells in E13 and E14 cultures (Figure 8.6D; $P < 0.01$).

8.3.6 Persistence of nestin expression

After 6 DIV differentiation, neurons, astrocytes and oligodendrocytes were present in all cultures. However, many cells in these cultures still express the intermediate filament protein, nestin. E12 cultures contained the highest percentage of nestin-positive cells (61.1 ± 2.8%; Figure 8.8A), with E13 (41.6 ± 3.1%; Figure 8.8B) and E14 (46.0 ± 2.0%; Figure 8.8C) cultures having significantly less nestin-positive cells (Figure 8.8D; $P < 0.01$). There was no significant difference between the percentage of nestin-positive cells in E13 and E14 cultures (Figure 8.8D).

8.3.7 Dopaminergic differentiation of VM neurospheres

Dopaminergic neurons were generated with much less frequency than all other cell types examined. Only E12 neurospheres consistently generated dopaminergic neurons upon differentiation. In E12 differentiated cultures, approximately 2% (1.7% ± 0.2%; Figure 8.9A, D) of the total cells were TH-positive. This means that of the neurons generated by E12 neurospheres (19.3 ± 0.6%), approximately 9% of these were dopaminergic. TH-positive neurons are rarely found in E13 differentiated cultures occurring at a frequency of one or two per coverslip (Figure 8.9B, D). Dopaminergic neurons were not found in E14 differentiated cultures (Figure 8.9C, D).

It has previously been suggested that dopaminergic neurons derived from neurospheres from E14 rat VM were not generated from a multipotential stem cell, but

were postmitotic neurons already present in the tissue at the time of dissection that survived in the neurosphere during the culture period (Ostenfeld et al. 2002). To address this issue, the expression of nurr1, an orphan nuclear receptor crucial for the development of midbrain dopaminergic neurons (Zetterstrom et al. 1997, Castillo et al. 1998), and of TH were examined using RT-PCR in neurospheres from E12, E13 and E14 rat VM after 6 DIV expansion as described above. No expression of nurr1 (Figure 8.10A) or TH (Figure 8.10B) was found in any of the neurospheres examined, suggesting that the dopaminergic neurons present after 6DIV differentiation of E12 neurosphere cultures arose from cells in the neurospheres which did not yet express these markers of differentiated dopaminergic neurons after 6 DIV of expansion.

8.4 Discussion

8.4.1 Age-related differences in the growth rates of rodent neurospheres

The effect of the age of the donor VM on the growth rates of the neurospheres in culture was apparent when sphere diameter and volume was measured at 2, 4 and 6 DIV during the expansion phase. Previously it has been shown that after 7 DIV expansion in medium containing 2% B27 with 20ng/ml EGF and 20ng/ml FGF-2, E14 rat VM neurospheres had a mean diameter of 260 ± 10μm (Ostenfeld et al. 2002). This is in agreement with the present finding that after 6 DIV expansion in culture, E14 neurospheres had a mean diameter of 189.9 ± 16.4μm. Neurospheres generated from E12 and E13, were significantly larger (in volume and diameter) than E14 neurospheres at all time points tested, with no significant differences observed between neurospheres generated from E12 and E13 VM. One explanation for this may be due to the timing of VM development. It has been proposed that by E14, many cells in the VM have undergone their last mitotic division and express TH (Altman and Bayer, 1981, Bayer et al. 1995). Thus at E14, the VM would contain fewer multipotential cells than at E12 or E13, which might explain the smaller size of neurospheres generated from this region at E14. Many cells isolated from the E12 VM and cultured *in vitro* for 24 hs have been shown to express nestin (Krieglstein et al. 2003; see Chapter 9), but less are nestin-positive in cultures of E13 or E14 VM after 1 DIV (O'Keeffe et al. unpublished observations).

8.4.2 Percentages of each cell type generated from VM neurospheres is dependent on the age of the donor VM

Having established that the growth rates of neurospheres are dependent on the age of the donor VM, the cell types present when these neurospheres were allowed to differentiate

were analysed. Removal of mitogens and exposure to serum has previously been shown to promote the differentiation of nestin-positive cells to mature phenotypes (Carpenter et al. 1999; Carvey et al. 2001; Storch et al. 2001). Neurospheres were dissociated, plated onto poly-D-lysine-coated dishes and allowed them to differentiate for 6 DIV. In agreement with previous studies, the dissociated cells gave rise to neurons, glia and oligodendrocytes (Svendsen et al. 1995, Ostenfeld et al. 2002). The numbers of each cell type generated however, was dependent on the age of the donor VM. Ostenfeld et al. (2002) found that expansion of E14 VM as neurospheres for 7 DIV followed by differentiation for 7DIV generated approximately 8% neurons as measured by TUJ-1-immunoreactivity. This agrees with the present finding that 6.9 ± 1.2% of the total cells were neurons. However in this study E14 neurospheres generated the lower numbers of neurons compared to E12 and E13 neurospheres, with neuronal differentiation increasing with decreasing age of donor (E12>E13>E14). The enhanced neuronal differentiation from E12 neurospheres may be explained by the fact that neurogenesis begins in the VM from E11 onwards (Hanaway et al. 1971; Lauder and Bloom 1974; Altman and Bayer 1981; Solberg et al. 1993; Bayer et al. 1995) and decreases as the VM matures. With increasing age, it is likely that the numbers of neuronal progenitors in the VM and thus in neurospheres generated from the VM decreases. This may explain why neurospheres generated from later aged VM show a progressive decrease in their ability to give rise to neurons, as more cells in these neurospheres may be nestin-expressing glial restricted precursors (GRPs).

In contrast to the lower generation of neurons from later aged VM, there is a progressive increase in the numbers of oligodendrocytes generated from neurospheres from later aged VM (E12<E13<E14). Again, these results agree with previous reports which showed that E14 VM rodent neurospheres generate approximately 10-20% oligodendrocytes after 7 DIV expansion followed by 7 DIV differentiation (Ostenfeld et

al. 2002). In the present study 13.5 ± 1.4% of the total cells generated from E14 neurospheres were oligodendrocytes. The lower generation of oligodendrocytes from neurospheres generated from VM of earlier ages may also be a reflection of the developmental status of the tissue at the time of harvesting for cell culture. It is possible that with increasing age, more cells in the VM are GRPs, which may explain the increased oligodendroglial differentiation from neurospheres of later ages. In E12 cultures, GFAP-positive cells constituted approximately 10% of the total cells. In E13 cultures, the percentage GFAP-positive cells was four-fold higher. There was no significant difference in the percentage of GFAP-positive cells between E13 and E14 cultures. This suggests that by E13, many more cells may be GRPs than at E12, explaining the increase in GFAP-positive cells generated from E13 and E14 neurospheres.

In contrast to the present study, Peaire et al. (2003) found that E12 VM expanded for 9 DIV in N2 containing medium supplemented with 10ng/ml FGF-2, and allowed to differentiate for three further days after mitogen withdrawal, gave rise to neurons (MAP2-positive), which represented 99% of the total cells and glia (GFAP-positive) which represented 1% of the total cells. No nestin or MBP expression was found in these cultures three days after mitogen withdrawal (Peaire et al. 2003). In the present study, only ~18% of the total cells in E12 differentiated cultures stained positively for MAP-2, with ~ 10% of the total cell population being GFAP-positive and 4% of the total cell population MBP-positive. A high level of nestin expression (~ 60% of total cells) remained in the E12 cultures after six days of differentiation. In agreement, Chow et al. (2000) generated free-floating neurospheres from E14 rat spinal cord, dissociated these to single cells after expansion and allowed these cells to differentiate for four or eight days in the presence of 10% FCS. In their cultures, approximately 70% of the total cells expressed nestin (Chow et al. 2000).

One possible explanation for the discrepancies between the above-mentioned studies, may be the differences in culture techniques used. Peaire et al. (2003) grew neurospheres as attached "microislands", while here and in other studies (Chow et al. 2000; Ostenfeld et al. 2002) neurospheres were grown as free-floating aggregates. Interestingly, Studer and co-workers also found a lower percentage of GFAP-positive cells (~3% when cells were differentiated in 10% serum, ~1% when cells were differentiated in serum free medium) in cultures generated from E12 rat expanded mesencephalic precursors grown as attached neurospheres for seven days in N2 medium supplemented with 10ng/ml FGF-2 (Studer et al. 1998), than was found in the present study (10%). In agreement with Peaire et al. (2003), no oligodendrocytes (Gal C-positive) were found in these cultures (Studer et al. 1998). So it is possible that method of expansion of mesencephalic progenitors may influence the potential of these cells to differentiate into mature cell types and this warrants further investigation.

The high level of nestin expression that remained in cultures of neurospheres of each of the three ages after seven days differentiation suggests that these cells retain multipotency and could be induced to differentiate into a required cell type. This means that factors capable of inducing multipotent stem cells to differentiate into a specific lineage may be useful in generating a required cell type. For example, retinoic acid has been shown to induce neuronal differentiation from rodent stem cells (Ruhnke et al. 2003). BMPs have been shown to enhance astrocyte differentiation from mouse telencephalic neural progenitor cells (Nakishima et al. 2001) while having a negative influence on oligodendrocyte differentiation (Yung et al. 2002). In contrast to the effects of BMPs, Shh has been shown to promote commitment to an oligodendroglial lineage (Yung et al. 2002). Detailed analysis of the intrinsic ability of neurospheres, generated from various CNS regions from different stages of development to differentiate into mature neural cell types will allow selection of the most suitable age of

donor tissue to use to generate a specific cell type. For example the present experiments suggest that E14 VM would be the most suitable age of those examined here if oligodendrocyte differentiation was required. Factors influencing this differentiation could then be examined for their ability to produce a population of oligodendrocytes for transplantation approaches to demyelinating disease (for review see Halfpenny et al. 2002).

The present study found that 1.7% of the total cells were TH-positive in the E12 cultures after six days of differentiation. This finding is in agreement with previous reports showing that 1.2% of the total cells in cultures of neurospheres from the nine week old human VM, were TH-positive following differentiation (Horiguchi et al. 2004). However to rule out the possibility that dopaminergic neurons derived from neurospheres from the E12 rat VM were not generated from a multipotential stem cell, but were postmitotic neurons already present in the tissue at the time of dissection that survived in the neurosphere during the culture period, we analysed the expression of nurr1 and TH in the neurospheres prior to differentiation. As no expression of Nurr1 and TH was seen in any of the neurospheres from each of the ages examined, the dopaminergic neurons must have differentiated from a cell not yet expressing these markers. This finding is supported by a report showing that no TH-positive neurons were present in neurospheres from the E14 rat VM after expansion under similar conditions as used in the present study (Storch et al. 2003). Previous reports have also showed that TH-positive neurons derived from rat mesencephalic neurospheres, produce and release dopamine, express voltage-gated sodium and potassium currents and fire action potentials which are functional characteristics of mature midbrain dopaminergic neurons (Storch et al. 2003). This data along with the present findings suggest that the production of dopaminergic neurons from expanded mesencephalic precursors is a viable method of producing dopamine neurons for transplantation approaches to PD,

however the age of donor tissue used is a crucial factor in determining the numbers of dopaminergic neurons that can be generated. Factors that improve dopaminergic neuronal differentiation can then be used to enhance this differentiation process (for reviews see Caldwell 2001; Arenas 2002)

Figure 8.1:

Photomicrographs showing representative cultures of neurospheres from (A) E12, (B) E13 and (C) E14 VM at 2 DIV. Graphical representation of (D) neurosphere diameter and (E) neurosphere volume from each of the ages examined. Data represents mean ± SEM taken from 3 independent experiments; 4 coverslips per experiment.

N.S. no significant difference

** $P<0.01$ compared to E12 and E13 values.

Scale bar = 100μm

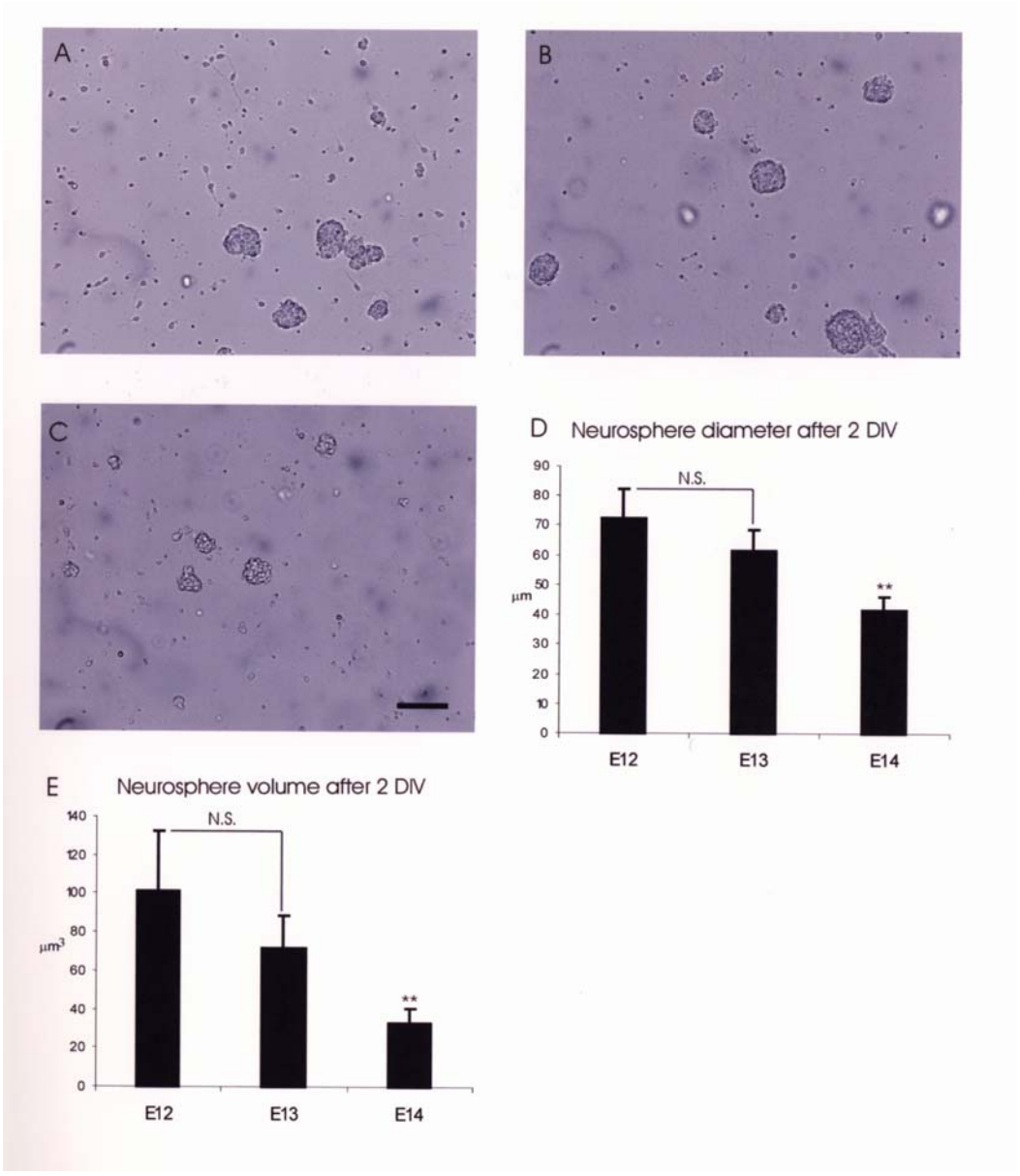

A
B
C
D Neurosphere diameter after 2 DIV
90
80
70
60
50
40
30
20
10
0
µm
N.S.
**
E12
E13
E14
E Neurosphere volume after 2 DIV
140
120
100
80
60
40
20
0
µm3
N.S.
**
E12
E13
E14

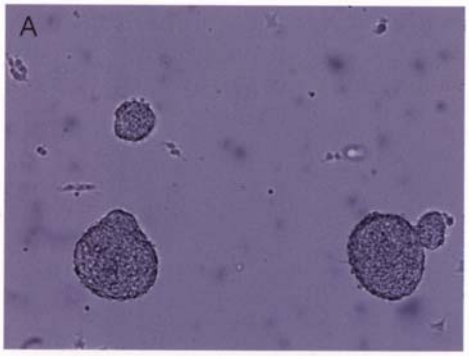
Figure 8.2:

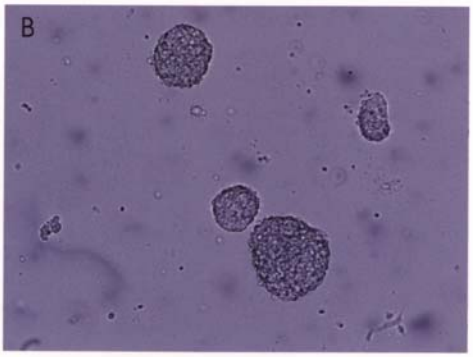
Photomicrographs showing representative cultures of neurospheres from (A) E12, (B) E13 and (C) E14 VM at 4 DIV. Graphical representation of (D) neurosphere diameter and (E) neurosphere volume from each of the ages examined. Data represents mean ± SEM taken from 3 independent experiments; 4 coverslips per experiment.

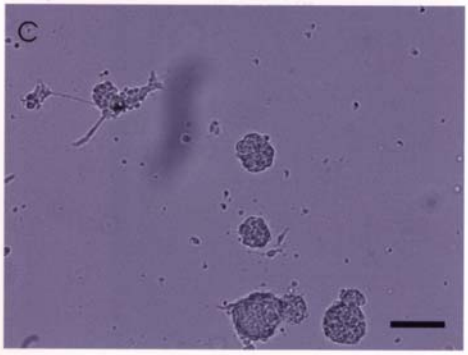
N.S. no significant difference

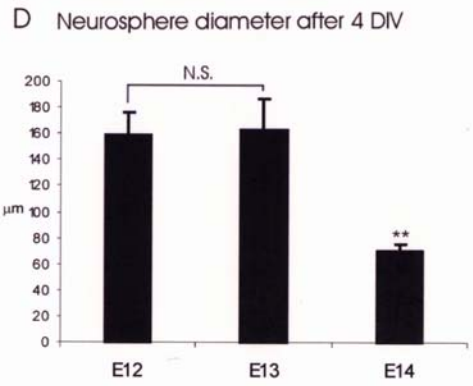
** $P<0.01$ compared to E12 and E13 values.

Scale bar = 100μm


A


B


C


D
Neurosphere diameter after 4 DIV
N.S.
200
180
160
140
120
µm 100
80
60
40
20
0
**
E12
E13
E14

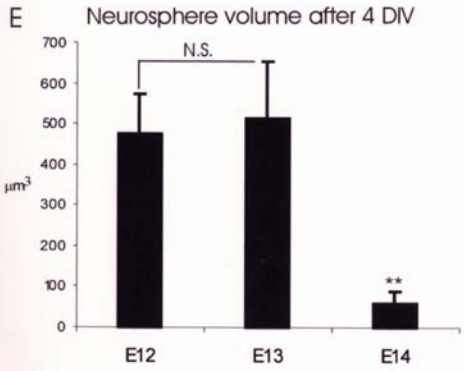

E
Neurosphere volume after 4 DIV
N.S.
700
600
500
400
µm3
300
200
100
0
**
E12
E13
E14

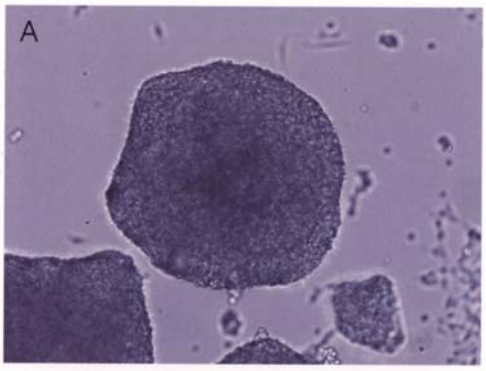
Figure 8.3:

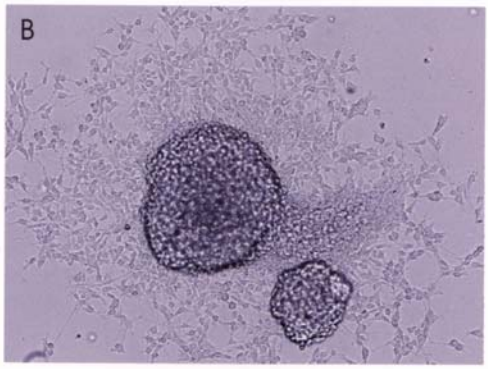
Photomicrographs showing representative cultures of neurospheres from (A) E12, (B) E13 and (C) E14 VM at 6 DIV. Graphical representation of (D) neurosphere diameter and (E) neurosphere volume from each of the ages examined. Data represents mean ± SEM taken from 3 independent experiments; 4 coverslips per experiment.

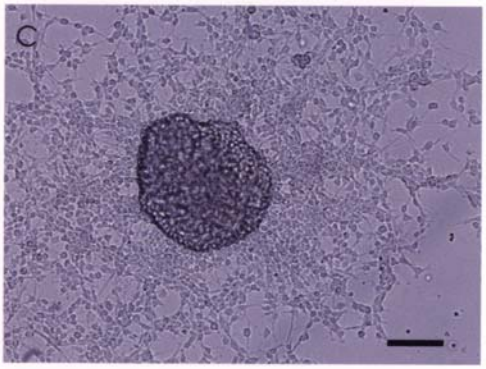
N.S. no significant difference

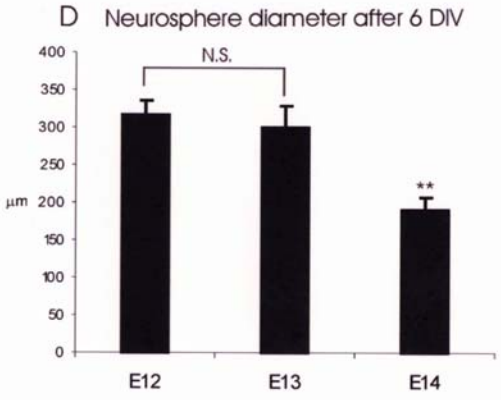
** $P<0.01$ compared to E12 and E13 values.

Scale bar = 100μm


A


B


C


D
Neurosphere diameter after 6 DIV
N.S.
**
µm
400
350
300
250
200
150
100
50
0
E12
E13
E14

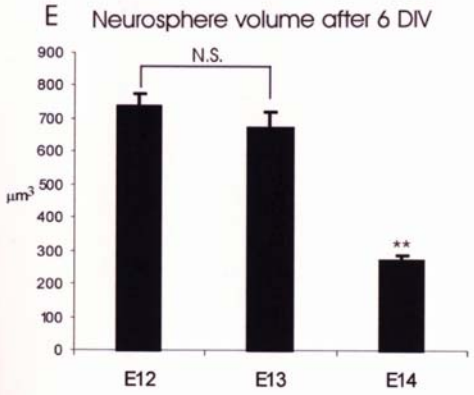

E
Neurosphere volume after 6 DIV
N.S.
**
µm3
900
800
700
600
500
400
300
200
100
0
E12
E13
E14

Figure 8.4:

Photomicrographs showing representative differentiated cultures plated from neurospheres from (A) E12, (B) E13 and (C) E14 VM expanded for 6 DIV. Cultures were immunocytochemically stained for TUJ. (D) Graphical representation of the percentage of total cells staining positively for TUJ in each of the three culture types. Data represents mean ± SEM taken from 3 independent experiments; 4 coverslips per experiment.

** P<0.01 compared to E12 values.

$ P<0.01

Scale bar = 100μm

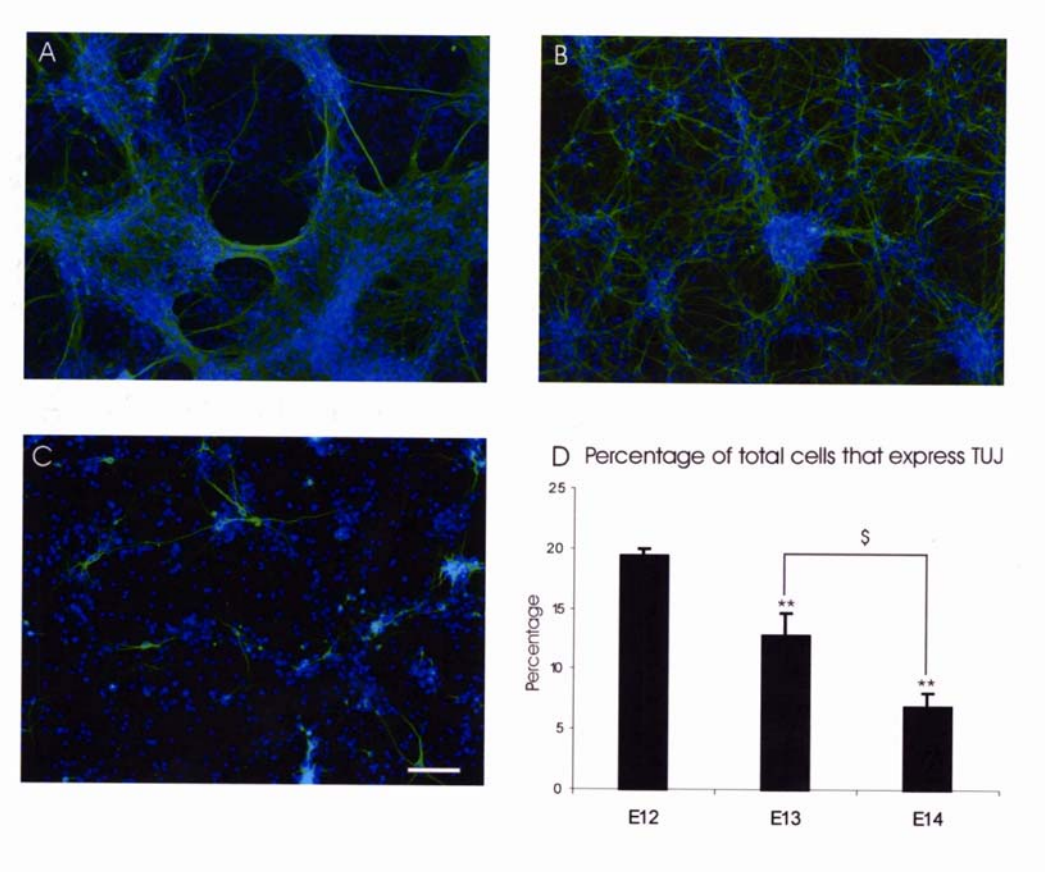


Figure 8.5:

Photomicrographs showing representative differentiated cultures plated from neurospheres from (A) E12, (B) E13 and (C) E14 VM expanded for 6 DIV. Cultures were immunocytochemically stained for MAP-2. (D) Graphical representation of the percentage of total cells staining positively for MAP-2 in each of the three cultures. Data represents mean ± SEM taken from 2 independent experiments; 4 coverslips per experiment.

** P<0.01 compared to E12 values.

$ P>0.01

Scale bar = 100μm

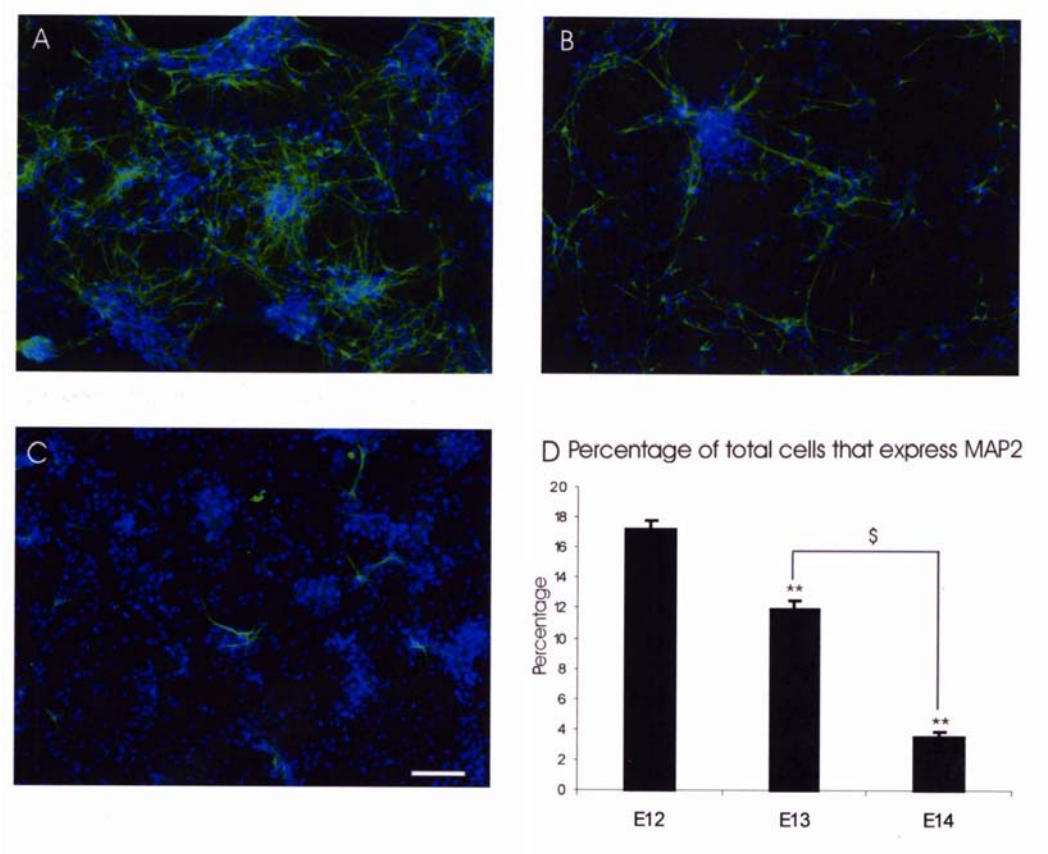


Figure 8.6:

Photomicrographs showing representative differentiated cultures plated from neurospheres from (A) E12, (B) E13 and (C) E14 VM expanded for 6 DIV. Cultures were immunocytochemically stained for GFAP. (D) Graphical representation of the percentage of total cells staining positively for GFAP in each of the three cultures. Data represents mean ± SEM taken from 3 independent experiments; 4 coverslips per experiment.

** P>0.01 compared to E12 values.

N.S. no significant difference

Scale bar = 100μm

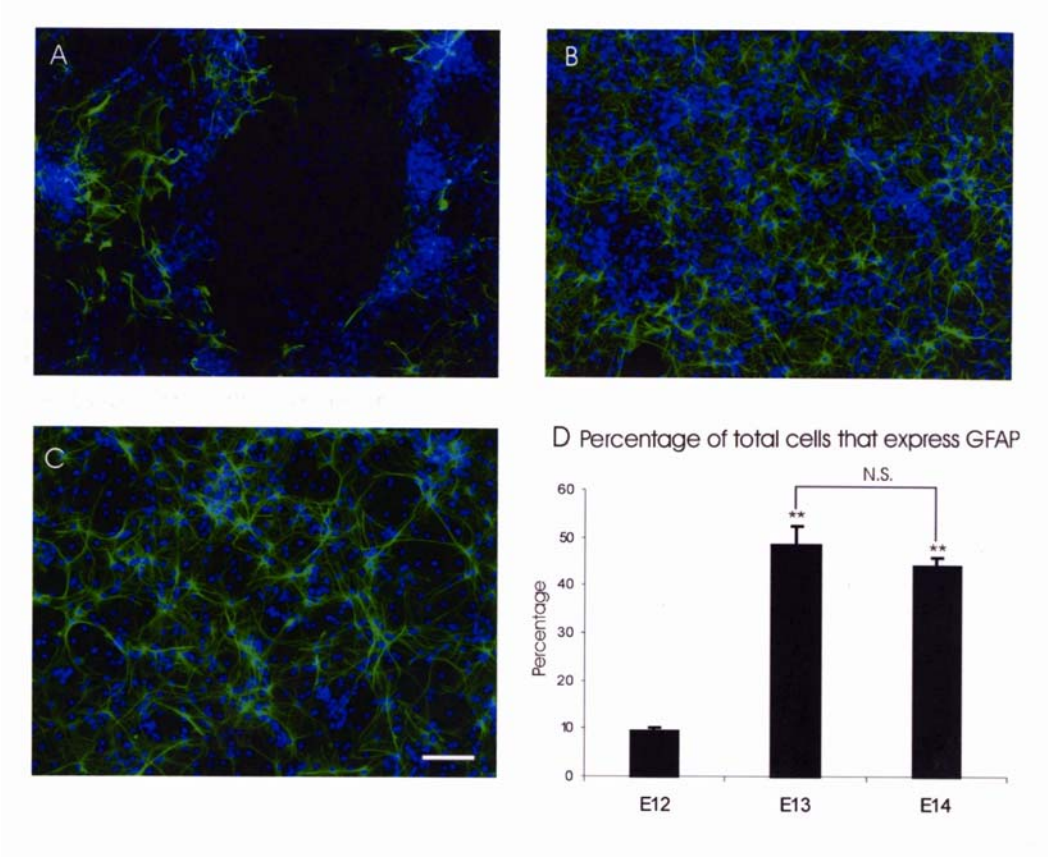


Figure 8.7:

Photomicrographs showing representative differentiated cultures plated from neurospheres from (A) E12, (B) E13 and (C) E14 VM expanded for 6 DIV. Cultures were immunocytochemically stained for MBP. (D) Graphical representation of the percentage of total cells staining positively for MBP in each of the three cultures. Data represents mean ± SEM taken from 3 independent experiments; 4 coverslips per experiment.

** P<0.01 compared to E12 values.

$ P<0.01

Scale bar = 100μm

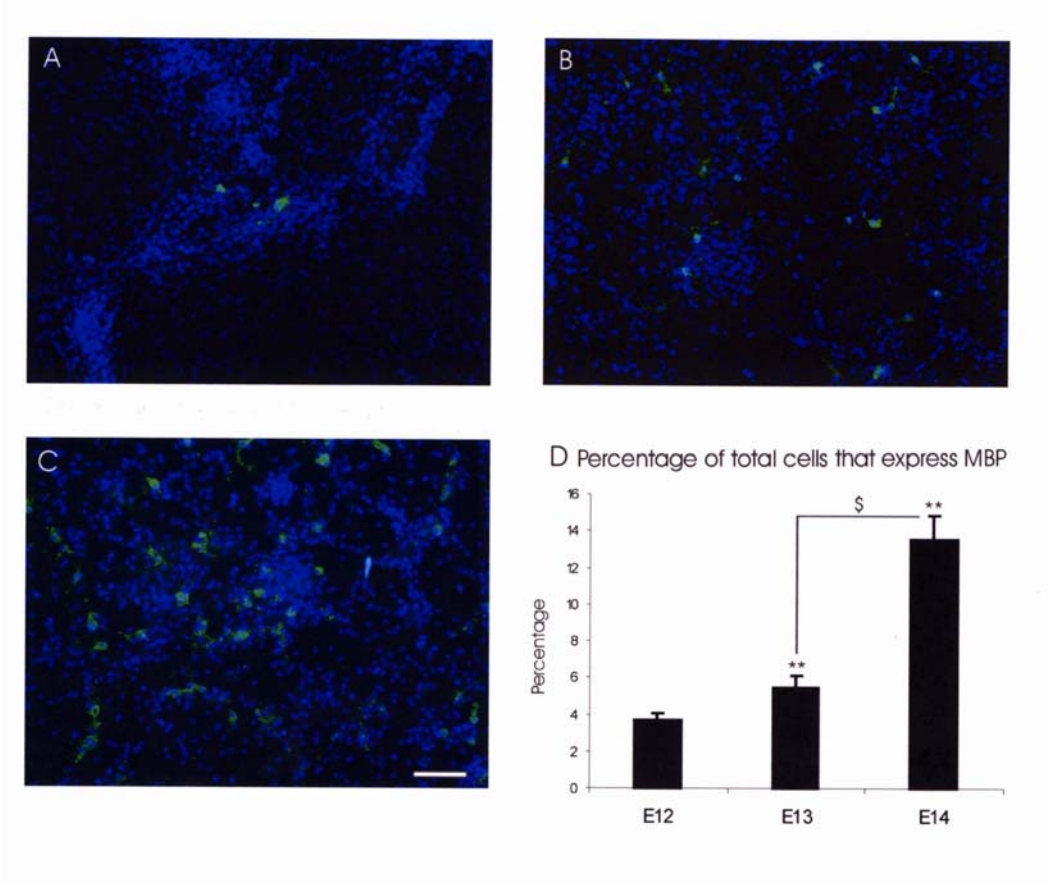


Figure 8.8:

Photomicrographs showing representative cultures of differentiated cultures plated from neurospheres from (A) E12, (B) E13 and (C) E14 VM expanded for 6 DIV. Cultures were immunocytochemically stained for nestin. (D) Graphical representation of the percentage of total cells staining positively for nestin in each of the three cultures. Data represents mean ± SEM taken from 2 independent experiments; 4 coverslips per experiment.

** P>0.01 compared to E12 values.

N.S. no significant difference

Scale bar = 100μm

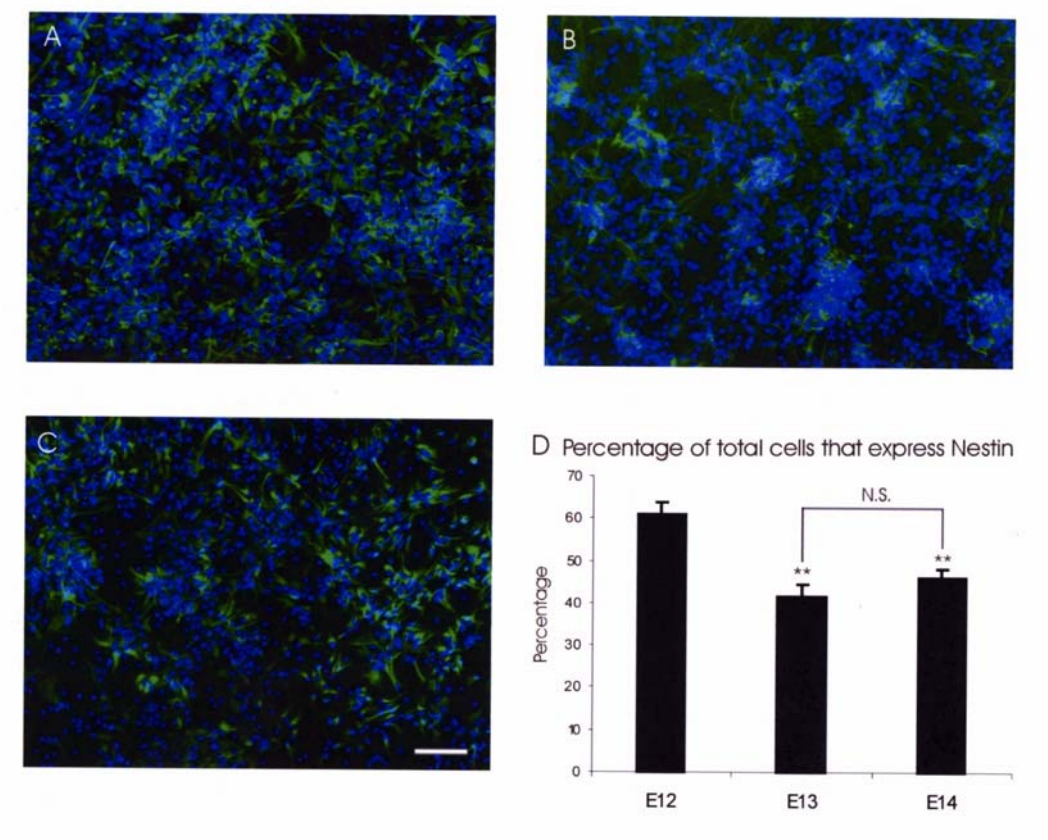


Figure 8.9:

Photomicrographs showing representative cultures of differentiated cultures plated from neurospheres from (A) E12, (B) E13, (C) E14 VM expanded for 6 DIV. Cultures were immunocytochemically stained for TH. (D) Graphical representation of the percentage of total cells staining positive for TH in each of the three cultures. Data represents mean ± SEM taken from 3 independent with 4 coverslips per experiment.

Scale bar = 100µm

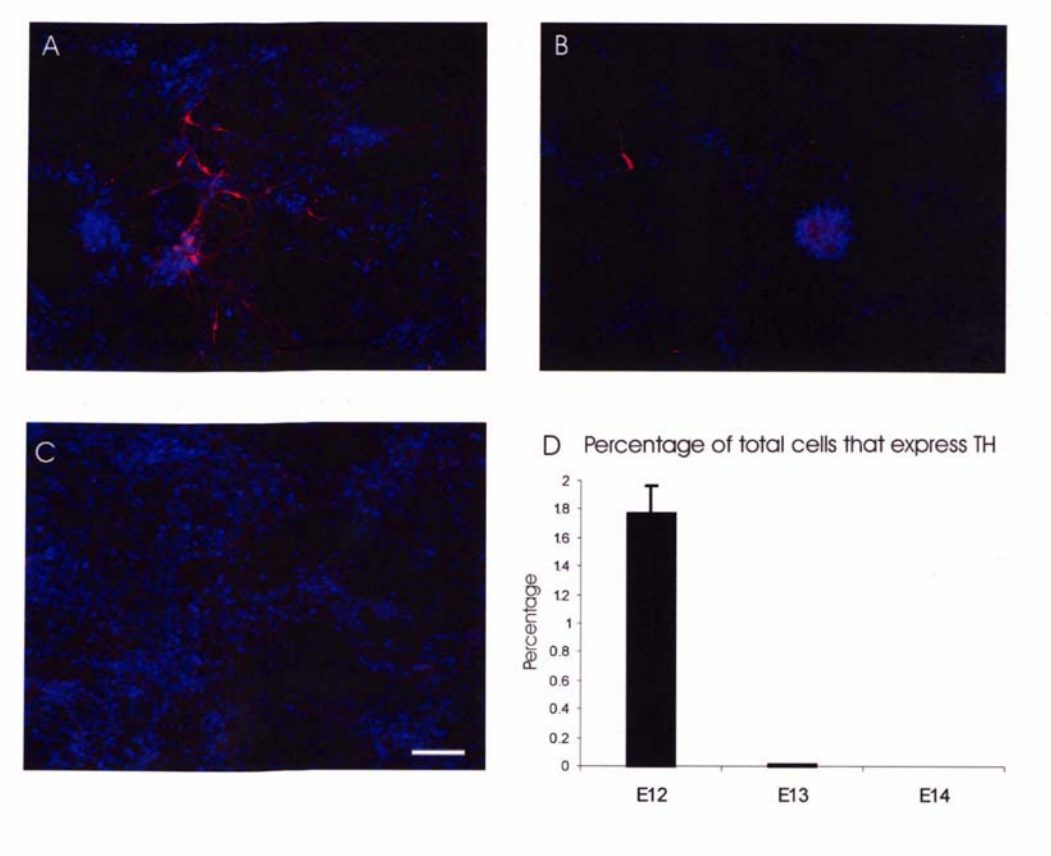
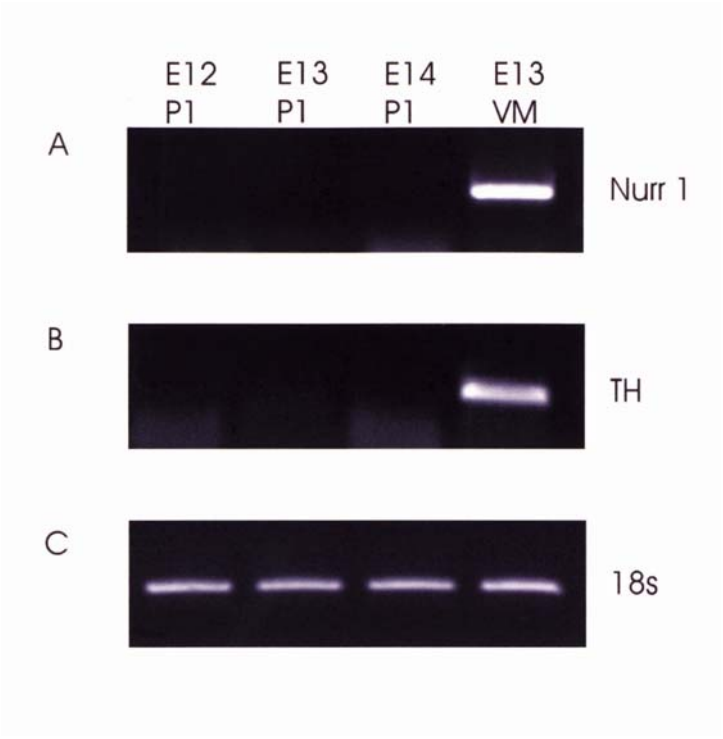


Figure 8.10:

RT-PCR for (A) nurr1 and (B) TH on cDNA from neurospheres prepared from E12, E13 and E14 rat VM after 6 DIV expansion. cDNA prepared from freshly-dissected E13 VM was used as a positive control. (C) 18s RNA was run to confirm the presence of intact of cDNA used in each of the samples. The demonstrated lack of nurr1 and TH expression in the E12 neurospheres shows that dopaminergic neurons derived from E12 neurospheres are as a result of differentiation from stem cells which did not yet express these markers after 6 DIV expansion rather than committed precursors or post-mitotic dopaminergic neurons that were contained in the sphere before differentiation.



9.0 THE ROLE OF GDF-5 AND BMPR-IB IN INDUCING DOPAMINERGIC DIFFERENTIATION IN CULTURES OF THE E12 RAT VM

9.1 Aims

- To examine the expression of GDF-5's receptors during dopaminergic neurogenesis in the VM.
- To characterise cultures of E12 VM after 1 DIV.
- To examine the effects of GDF-5 on cultures of E12 VM after 1 DIV.
- To examine the effect of GDF-5 on gene expression of BMPR-II, BMPR-Ia and BMPR-Ib, Ror2, Nurr1 and TH in E12 expanded VM precursor cells.
- To examine the role of BMPR-Ib signalling in TH induction by GDF-5.

9.2 Introduction

The VM contains the developing dopaminergic neurons of the future SN, those that degenerate in PD. Unravelling the factors that play a role in the development of these neurons is important if efficient generation of mature functional dopaminergic neurons from stem cells for use in transplantation approaches to PD is to become a reality. In the rat VM, dopaminergic neurons proliferate between E11 and E15, undergoing their final mitotic divisions on E14-15 (Hanaway et al. 1971; Lauder and Bloom 1974; Altman and Bayer 1981; Solberg et al. 1993; Bayer et al. 1995). In the rat, axons start to invade the striatum at E16 and continue to grow pre- and post-natally (Unsicker et al. 1996). VM dopaminergic neurons are generated in the midbrain/hindbrain boundary under the influence of two major signalling centres, the isthmus and the floor plate (Ye et al. 1998). Among the secreted molecules known to regulate the development of VM dopaminergic neurons, two of the most important are Shh and FGF-2-8 (Ye et al. 1998). TGF-β2, TGF-β3 and the TGF-β type II receptor have been shown to be expressed in the developing rat VM at E12.5 and are required for the induction of dopaminergic neurons by Shh (Farkas et al. 2003). A number of transcription factors have also been shown to be crucial for the development of VM dopaminergic neurons. These include Nurr-1 (Zetterstrom et al. 1997; Castillo et al. 1998), Pitx-3/Ptx-3 (Smidt et al. 1997), Lmx-1b (Asbreuk et al. 2002) and the En genes, En-1 and En-2 (Simon et al. 2001). Recently further genes which are involved in this process have been identified (Thuret et al. 2004).

Although GDF-5 expression peaks on E14 during the development of the rat VM, it is also expressed at E12 (O'Keeffe et al. 2004a), which is the day the first dopaminergic neurons in the VM express TH (Hanaway et al. 1971; Lauder and Bloom 1974; Altman and Bayer 1981; Solberg et al. 1993; Bayer et al. 1995). GDF-5 has been

shown to increase the numbers of TH neurons in cultures of E14 rat VM (O'Keeffe et al. 2004b; see Chapter 6) and to improve the morphological development of these neurons (O'Keeffe et al. 2004b; see Chapter 6). However, there is no information on whether GDF-5 is involved in the induction of a dopaminergic fate in early (E12) rat VM. The present set of experiments examined the ability of GDF-5 to induce dopaminergic differentiation from neural progenitor cells from the VM. Also the involvement of BMPR-Ib in this induction was examined.

9.3 Results

9.3.1 BMPR-II, BMPR-Ia and BMPR-Ib are expressed in the rat VM at E12, E13 and E14

Previous experiments have shown that GDF-5 is expressed in the E14 rat VM (O'Keeffe et al. 2004a; see Chapter 5), improves the morphological development of E14 rat VM dopaminergic neurons *in vitro* (O'Keeffe et al. 2004b; see Chapter 6) and increases dopaminergic neuronal differentiation in E14 rat VM cultures (see Chapter 7). Furthermore GDF-5 has been shown to be expressed in the rat VM at E12 (O'Keeffe et al. 2004a; see Chapter 5). However no function has been described for GDF-5 on early VM precursors at this stage of development (E12). To confirm that the appropriate receptors for GDF-5 were present in the VM at E12, RT-PCR for BMPR-II, BMPR-Ia and BMPR-Ib was carried out on cDNA from the rat VM at E12, E13 and E14. The pieces of VM were dissected from the area immediately adjacent to the ventral midline and rostral to the isthmus. Every effort was made to remove all contaminating meningeal tissue. The receptors for BMPR-II, BMPR-Ib and BMPR-Ia were detected from E12 through to E14 (Figure 9.1A, B, C respectively). 18s RNA was run as a positive control (Figure 9.1D) and reactions without the RT enzyme were carried out to ensure that no contaminating genomic DNA was present (Figure 9.1E). Immunocytochemistry for BMPR-II, BMPR-Ia and BMPR-Ib was performed on cultures of E12 rat VM after 1 DIV to confirm the RT-PCR results. Widespread expression of all three receptors was seen in these cultures (Figure 9.2A-C). Negative control cultures, which omitted the primary antibody from the staining procedure, consistently displayed no staining (Figure 9.2D). This finding that the appropriate receptors are present in the rat VM at E12, along with the previous report showing that

GDF-5 is expressed in the rat VM at E12 (O'Keeffe et al 2004a; Chapter 3), suggests that GDF-5 plays a role at this stage of development of the rat VM.

9.3.2 GDF-5 increases numbers of TH neurons in E12 VM cultures

To analyse the role of GDF-5 in the rat VM at E12, cultures of E12 rat VM were grown for 1 DIV. Initially the expression of neuronal, glial and precursor cells markers in the cultures was characterised. After 1 DIV 56.1 ± 4.3% of the total cells were TUJ-1-positive (Figure 9.3A, E), 1.9 ± 0.1% of the total cells were TH-positive (Figure 9.3A, E) and 26.8 ± 1.3% of the total cells were BrdU-positive (Figure 9.3C, E). A high proportion of the cells (approximately 40%) expressed nestin after 1 DIV (Figure 9.3B). No GFAP or MBP expression was found in these cultures (Figure 9.3D).

Addition of 10ng/ml GDF-5 to these cultures at the time of plating induced a significant increase in the numbers of TH-positive cells after 1DIV when compared to controls ($P<0.05$; Figure 9.4A, B, C). This increase in TH-positive cell number could have been due to a) induction of TH expression in uncommitted precursors, b) proliferation of TH-positive cells which were already in the culture and/or c) increased survival of TH-positive cells in these cultures.

To address the possibility that GDF-5 treatment increased survival or prevented cell death in these cultures, the degree of cell death in these cultures was measured by counting pyknotic (apoptotic) nuclei using DAPI staining. There was no significant difference in the number of apoptotic cells or in the total number of cells in control cultures compared to GDF-5 treated cultures (Figure 9.5A, B respectively), which suggested that GDF-5 did not influence the survival of cells in these cultures. To examine whether GDF-5 may have stimulated the proliferation of TH-positive cells in these cultures thus increasing their number, cell proliferation was examined. Repeated

experiments found no cells which were doubled-labelled with proliferating cell nuclear antigen (PCNA) and TH, which showed that GDF-5 did not stimulate proliferation of TH-positive cells in these cultures (Figure 9.6A, B). Furthermore there was no significant difference in the numbers of BrdU-positive cells in control cultures compared to GDF-5-treated cultures, confirming that GDF-5 did not have an effect on cell proliferation in these cultures (Figure 9.6C). These experiments suggest that the increase in TH-positive cell numbers induced by GDF-5 treatment was as a result of induction of TH expression in progenitor cells.

9.3.3 GDF-5, BMP2 and Shh induce Nurr1 and TH in VM NPCs

To further examine the possibility that GDF-5 induces TH expression in progenitor cells, cultures of NPCs were established from the E12 rat VM. The reason for using cultures of NPCs was two-fold. Firstly, more cells are available in these cultures than primary cultures of E12 rat VM for subsequent RT-PCR analysis. Secondly, it was important to determine whether GDF-5 can induce Nurr1 and TH expression in proliferating NPCs, since these cells (and not primary cultures of E12 VM) have been proposed as a potential source of cells for generating dopaminergic neurons for the treatment of PD.

The experimental protocol involved culturing cells for 6DIV in 2% B27, 20ng/ml EGF/FGF-2 followed by treatment with GDF-5 and the other factors for 24 hs (Figure 9.7). During the 6DIV of expansion, single cells proliferated into large cultures that were attached to the bottom of each well (Figure 9.8A). Although other groups have performed similar experiments using FGF-2 alone (Panchision et al. 2001), in the present set of experiments it was found that the cells did not proliferate effectively in either FGF-2 or EGF alone (Figure 9.8B, C). Thus both EGF and FGF-2 were used in this set of experiments.

After 6DIV expansion, cultures were treated with GDF-5, BMP-2, BMP-2 and Shh or BMP-2 and GDF-5 for 24 hs. RT-PCR was used to analyse gene expression in these cultures. Densitometic analysis of the RT-PCR data revealed that treatment with GDF-5, BMP2 or BMP2 and Shh increased the expression of BMPR-Ib, BMPR-Ia, nurr1 and TH (Figure 9.9 A, B, D, E respectively) when compared to control cultures. BMP-2 and GDF-5 co-treatment down-regulated the expression of BMPR-Ib, BMPR-Ia, Nurr1 and TH when compared to the controls (Figure 9.9A, B, D, E respectively).

BMP-2 has previously been shown to upregulate the expression of Msx-1, a gene normally expressed in the most dorsal domain of the spinal cord and forebrain (Grove et al. 1998; Bang et al. 1999); this up-regulation was prevented by treatment with Shh (Panchision et al. 2001). Thus the expression of Msx1 in the cultures in response to the aforementioned treatments was examined in the present study. BMP-2 did not induce the expression of Msx1 (Figure 9.9F). As a positive control, Msx1 was amplified from cDNA prepared from the adult rat brain, indicating that the amplification parameters for Msx1 were optimal.

The tyrosine kinase receptor Ror2 (see Chapter 7) was recently found to be a receptor for GDF-5 (Dr. Prezmko Tylanowski, personal communication) and Wnt-5a (Oishi et al. 2003). In the present study, no expression of Ror-2 was found in control cultures. Treatment with GDF-5 or BMP-2 and Shh co-treatment induced the expression of Ror2 in these cultures, whereas BMP-2 alone did not (Figure 9.9C). Co-treatment with BMP-2 abolished the induction of Ror-2 induced by GDF-5 alone (Figure 9.9C).

9.3.4 GDF-5 increases numbers of TH-positive cells in differentiated VM NPCs

Although GDF-5 increased the expression of nurr1 and TH in proliferating VM NPCs, it was not known if increased numbers of dopaminergic neurons could be generated when

these cells were induced to differentiate. This is important to demonstrate if GDF-5 is to be used to induce dopaminergic neuronal differentiation from expanded NPCs for transplantation approaches to PD. As a pilot study to assess this, proliferating VM NPCs from the E12 rat VM were differentiated with or without GDF-5 for 6 DIV. In GDF-5-treated cultures (Figure 9.10A) there appeared to be increased numbers of dopaminergic neurons compared to control cultures (Figure 9.10A), however further experiments are necessary to confirm and quantify this finding.

9.3.5 Signalling through BMPR-Ib is required for increases in Nurr1 and TH expression by BMP-2

In order to examine the role of BMPR-Ib (the type I receptor shared by both GDF-5 and BMP-2), in increasing the expression of nurr1 and TH by GDF-5 and BMP-2, blocking antibodies to BMPR-Ib were used to inhibit ligand binding to the receptor. As GDF-5 induced the expression of Ror2 (see above), BMP-2 was used instead of GDF-5 in this experiment. RT-PCR was used to analyse the expression of nurr1 and TH in the treated cultures. Densitometric analysis of the RT-PCR data showed that treatment of the cultures with BMP-2 increased the expression of nurr1 (Figure 9.11A, D) and TH (Figure 9.11B, E) when compared to control cultures. Treatment with blocking antibodies to BMPR-Ib abolished this effect (Figure 9.11A-E), suggesting that signalling through BMPR-Ib is required for induction of nurr1 and TH by BMP-2 (and presumably GDF-5) in E12 rat VM progenitor cells.

9.4 Discussion

9.4.1 BMP receptors are expressed during the period of dopaminergic neurogenesis in the rat VM.

In the developing rat VM, BMP-2, -4 and –6 (Jordan et al. 1997) and GDF-5 (O'Keeffe et al. 2004a) are expressed at E13, E14 and E15. In the mouse VM, GDF-1 has been shown to be expressed from E9.5 onwards (Wall et al. 2000). This suggests that dopaminergic neurons are subject to BMP/GDF signalling during their development. The receptors for GDF-5 have been shown to be BMPR-II and BMPR-Ib (Nishitoh et al. 1996). More recently, Ror2 has been identified as a novel receptor for GDF-5 (Dr. Prezmko Tylanowski, personal communication). However it has previously been reported by *in situ* hybridisation studies that BMPR-Ia is expressed in all proliferative regions of the developing mouse neural tube after gastrulation, whereas BMPR-Ib expression is restricted to the dorsal domain of the neural tube (DeWulf et al. 1995; Panchision et al. 2001). In the present study, expression of all three BMP receptors was found in the developing rat VM. The discrepancy between these studies could possibly be explained by the varying sensitivities of the techniques used. For example, a previous study has shown widespread expression of GDF-15 in various regions of the rat brain at P1 that is detectable by RT-PCR and Western bloting, whereas *in situ* hybridisation detected transcripts for GDF-15 only in the choroids plexus (Strelau et al. 2000). As such the findings of the present study taken together with those of previous studies (DeWulf et al. 1995; Panchision et al. 2001), suggest that, while BMPR-Ib expression may be at its highest in the dorsal domain of the neural tube, it is also expressed in the ventral midbrain at lower levels. This suggests that it plays a role(s) during VM development (see below). The present set of experiments provide the first direct evidence that cells in the E12 rat VM express all three BMP receptors.

9.4.2 GDF-5 increases the numbers of TH-immunopositive cells in E12 rat VM cultures

Cultures of E12 rat VM were used to examine the effect of GDF-5 on the numbers of TH-positive cells in tissue taken at an early stage of dopaminergic neuron development. A similar culture system has been used previously to examine the effects of TGF-β on rat dopaminergic neurons at E12 (Farkas et al. 2003). Initially the cultures were characterised in order to determine the types of cells present after 24 hs *in vitro*. Slight differences were observed between culture composition in the present experiment and that of Farkas and co-workers. In the present experiments, 56.1% of the cells in the culture expressed TUJ-1, compared to 34.6% in the Farkas study. Farkas et al. also observed a higher percentage of TH-positive cells; 8.7% compared to 1.9% in the present set of experiments. These discrepancies may be due to the accuracy of the dissection, which may have differed between the experiments, and would greatly influence the relative percentages of cells present in the cultures. Many cells in the present set of experiments were nestin-positive and thus probably represented neural progenitor cells. The high degree of cells that were BrdU-positive indicates that many cells in these cultures were still proliferating and had not undergone their final mitotic division, which is thought to occur at about E14 in dopaminergic neurons of the rat VM (Hanaway et al. 1971; Lauder and Bloom 1974; Altman and Bayer 1981; Solberg et al. 1993; Bayer et al. 1995).

Previously it has been shown that treatment of E12 rat VM cultures for 24 hs with 1ng/ml TGF-β resulted in a two-fold increase in the numbers of TH-positive neurons (Farkas et al. 2003). In the present set of experiments, GDF-5 resulted in an increase of 1.6-fold of these neurons. In agreement with the Farkas study, this increase was not due to an increase in total cell number or proliferation of TH-positive cells as no

cells which were stained positively for both TH and PCNA could be found and GDF-5 did not increase the numbers of cells that incorporated BrdU. Taken together, the data presented here show that the GDF-5-mediated increase in the numbers of TH-positive neurons in cultures of E12 rat VM is caused by induction of a dopaminergic phenotype in progenitor cells.

9.4.3 GDF-5 and BMP-2 induce nurr1 and TH in NPCs from E12 rat VM

In an attempt to try and provide direct evidence that GDF-5 can induce a dopaminergic phenotype in progenitor cells, cells from the E12 rat VM were expanded using 20ng/ml EGF and 20ng/ml FGF-2 for 6 days, followed by treatment with the aforementioned factors for 24 hs in the presence of EGF and FGF-2. A previous study examined the effect of BMP-2 treatment on E12 rat VM cells expanded in a similar manner (Panchision et al. 2001). In that study, no BMPR-Ib expression was found in the cultures after 6 DIV. BMP-2 induced the expression of BMPR-Ib and of the dorsal identity gene, Msx1, and this induction could be blocked by Shh (Panchision et al. 2001). In the present experiment, it was found that BMPR-Ib was expressed in these cultures after 6 DIV and that BMP-2 did not induce the expression of Msx1 but instead up-regulated nurr1 and TH. These results suggest that, instead of acting as a dorsalising factor as previously suggested (Panchision et al. 2001), BMP-2 acts as a ventralising factor. One possible reason for the discrepancy between these experiments may be due to the presence of EGF in the present study. In the experiments of Panchision and co-workers, FGF-2 alone was used as the mitogen, whereas in the present set of experiments, both EGF and FGF-2 were used as mitogens because either factor alone did not result in efficient expansion of the cells.

EGF has been shown to activate a p42/44 Erk-mediated pathway, which phosphorylates specific serine residues in the linker region of smad-1, preventing BMP-

induced nuclear accumulation of smad-1 (Kretzschamer et al. 1997). So in the presence of EGF, signalling through a smad-dependant mechanism is thought to be in-activated. This fact may provide a possible explanation for the discrepancy seen between the Panchision study and the present study. As Panchision and co-workers did not have EGF present in their cultures, it is possible that the BMP-2-mediated induction of a dorsal phenotype seen in their study was dependant on a smad-mediated signal. In the present experiment the presence of EGF (which is thought to inactivate smad signalling) suggests that the induction of a ventral phenotype may be achieved by a smad-independent mechanism. One possibility is that GDF-5 could have an effect through activation of members of the MAPK kinase family. In support of this hypothesis, a recent report found that GDF-8 activated a p38 MAPK signal transduction pathway through TGF-β-activated kinase 1 (TAK1) that induced changes in gene expression that were independent of smad signalling (Philip et al. 2004 *in press*). Previous reports have also shown BMP-5-mediated induction of certain genes in the developing limb occurs through a smad-dependant mechanism, while the induction of other genes occur through a smad-independent mechanism (Zuzarte-Luís et al. 2004). Furthermore BMPs and GDFs have been reported to activate signalling pathways other than the smad pathway. For example GDF-5, BMP-2 and GDF-15 can activate MAPK kinase signalling cascades, including P38 and Erk-MAPK kinase (Nakamura et al. 1999; Hassel et al. 2003; Subramaniam et al. 2003, respectively). The precise signalling events leading to the increase in nurr1 and TH expression found in the present study remain to be established, but it is clear that the functions of BMP-2 during mesencephalic development are not clearly understood (Panchision et al. 2001). These experiments also suggest the possibility that EGF may act as a "switch", by allowing the same ligand (BMP-2) to have different effects depending on the (possibly) graded expression of EGF in the developing neural tube.

Panchision and co-workers reported that the actions of BMP-2 in cultures of the E12 rat VM could be blocked by Shh. Shh is expressed in the floor plate and notochord, is secreted from the floor plate, and induces neurons in the midbrain to adopt a dopaminergic fate (Hynes et al. 1995a, 1995b). Shh has been shown to require TGF-β (which activates smad-signalling) for this effect (Farkas et al. 2003). In the present set of experiments, treatment with Shh and BMP-2 in combination had the same effect as treatment with BMP-2 alone; that is upregulation of nurr1 and TH expression. This suggests that Shh does not block the effects of BMP-2 to induce a dopaminergic (ventral) phenotype, in contrast to its blocking effect on BMP-2 induction of a dorsal phenotype in ventral cells.

The expression of BMP-2 in the E13 rat VM (Jordan et al. 1997) and GDF-5 (O'Keeffe et al. 2004a) from E12 on in rat VM is surprising given that in the present set of experiments BMP-2 and GDF-5 alone increase nurr1 and TH-expression but when added together to the culture they downregulate the expression of nurr1 and completely switch off the expression of TH. This may be due to competition for binding to BMPR-Ib. To explain how such a finding could be reconciled with the in vivo scenario is difficult however it is possible that these factors may act as negative influencers of the others function. In order to examine this the amounts of BMP-2 and GDF-5 relative to each other should be compared during rat VM development.

Ror2 (along with Ror1) was cloned from a human neuroblastoma cell line (Masiakowski and Carroll, 1992). Recently, Ror2 has been identified as a receptor for GDF-5 (Dr. Prezmko Tylanowski personal communication) and for Wnt-5a (Oishi et al. 2003). In the present set of experiments, GDF-5, but not BMP-2, induced the expression of Ror2, suggesting that GDF-5 may facilitate signalling by itself and by Wnt-5a in the developing VM, by providing an appropriate receptor. Previously it has been shown that the expression of Wnt-5a peaks on E11.5 in the rat VM (Castelo-

Branco et al. 2003) which correlates with the onset of GDF-5 expression in the VM found in the present study (O'Keeffe et al. 2004a; see Chapter 5). Even though GDF-5 and Wnt-5a have no additive effects in increasing TH-positive cell numbers in cultures of E14 rat VM (see Chapter 6), it is possible that GDF-5 provides a niche for Wnt-5a signalling to occur by inducing a receptor which Wnt-5a can act on. As Ror2 was not induced by BMP-2 treatment but was induced by BMP-2/Shh co-treatment, this suggested that Shh alone can also induce the expression of Ror2. However any role for Ror2 in dopaminergic differentiation or induction remains to be established. The increased numbers of TH-positive neurons following differentiation of GDF-5-treated cultures suggests that GDF-5 may be useful in inducing dopaminergic differentiation from expanded mesencephalic precursor cells for transplantation approaches to PD.

9.4.5 Requirement for BMPR-Ib in BMP-2-mediated nurr1 and TH induction

In the present set of experiments, both BMP-2 and GDF-5 increased the expression of nurr1 and TH in cultures of NPCs from the E12 rat VM. In order to achieve intracellular smad activation, phosphorylation of type-I receptors by type-II receptors following ligand binding is necessary (for review see ten Dijke. et al. 2000). Of the type-I receptors, BMP-2 and GDF-5 can both act through BMPR-Ib (for review see Mehler et al. 1997). As such it was likely that signalling through BMPR-Ib was involved in the induction of nurr1 and TH by BMP-2 and GDF-5. As GDF-5 induced Ror2 expression whereas BMP-2 did not, BMP-2 was the ligand chosen to use in the experiments designed to examine this question, because the use of GDF-5 would have introduced another receptor, of unknown function, into the experiment. Blocking antibodies to BMPR-Ib abolished the BMP-2 induced increase in nurr1 and TH expression, which suggested that signalling through BMPR-Ib is required for the increase in nurr1 and TH induced by BMP-2 (and presumably by GDF-5, although this

will need to be shown directly). Previous reports have shown that BMPR-Ib signalling is involved in the generation of dorsal identity within the neural tube (Panchision et al. 2001). The present set of experiments provide the first evidence that BMPR-Ib can also be involved in the generation of ventral cell types in the rat midbrain.

Figure 9.1:

RT-PCR analysis of the expression of BMP receptors (A) BMPR-II, (B) BMPR-Ib, (C) BMPR-Ia in freshly dissected E12, E13 and E14 rat VM tissue. (D) 18s RNA was run to confirm the presence of intact of cDNA used in each of the samples. (E) An RT minus group was also used for the 18s RNA to confirm that there was no contamination of the cDNA samples with genomic DNA.

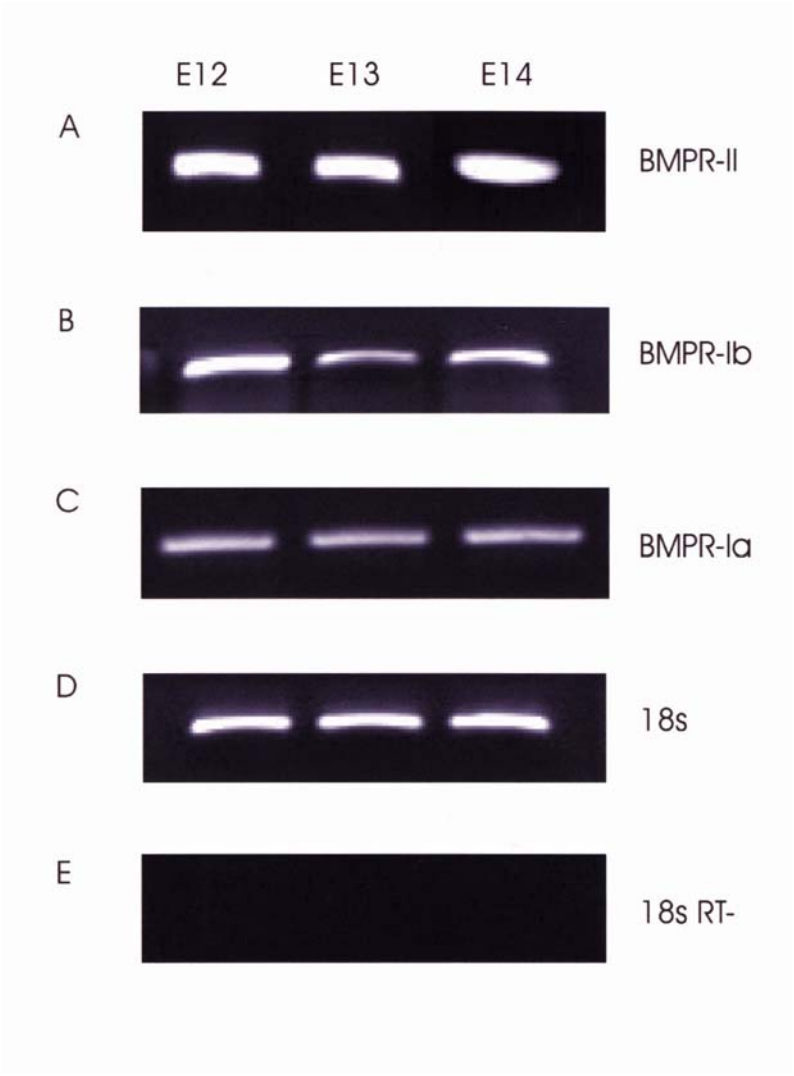


Figure 9.2:

Immunocytochemical analysis of the BMP receptors, (A) BMPR-II, (B) BMPR-Ia and (C) BMPR-Ib in E12 rat VM cultures after 1 DIV. (D) shows a negative control, i.e. a cultures that received no primary antibody.

Scale bar = 50μm

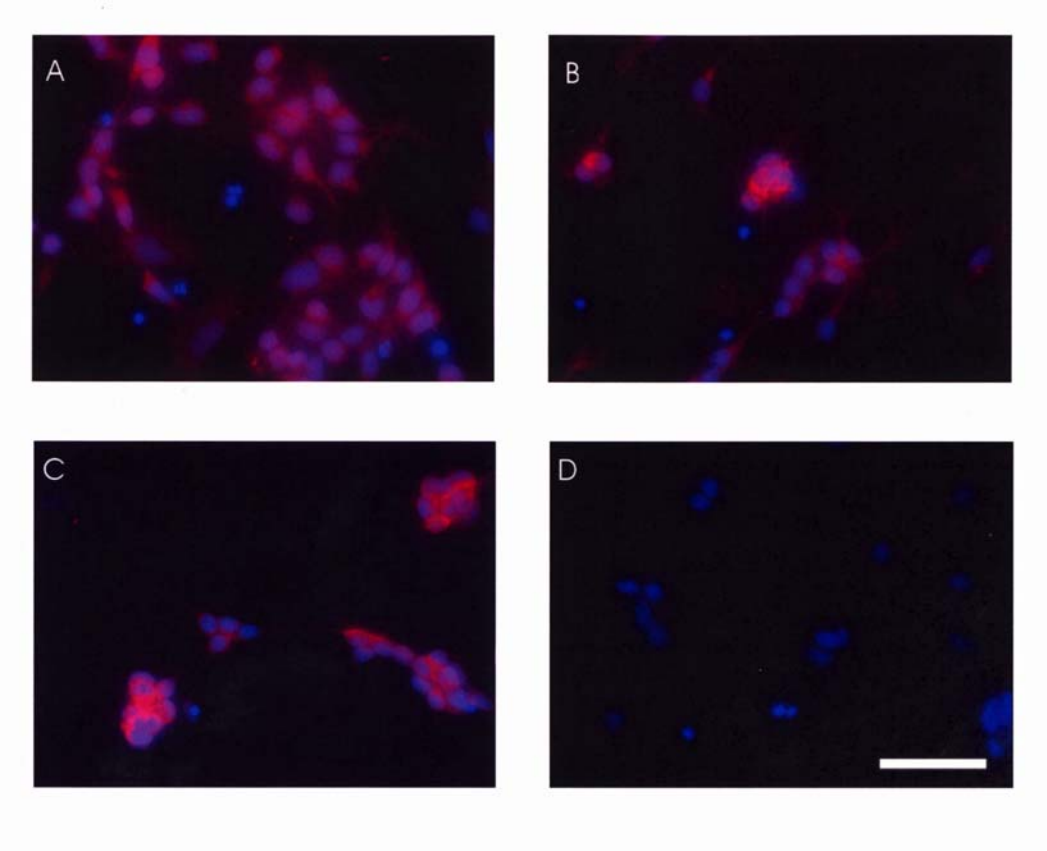


Figure 9.3:

Photomicrographs showing representative cultures of E12 rat VM grown for 1 DIV. Cultures were immunocytochemically stained for (A) TH (red), TUJ (green) and DAPI (blue), (B) nestin (green) and DAPI (blue), (C) BrdU (green) and DAPI (blue) and (D) GFAP (red), MBP (green) and DAPI (blue). (E) Graphical representation of showing the percentage of total cells in E12 VM cultures after 1 DIV expressing TUJ, TH and BrdU. Data represent mean ± SEM taken from three independent experiments.

Scale bar = 100μm

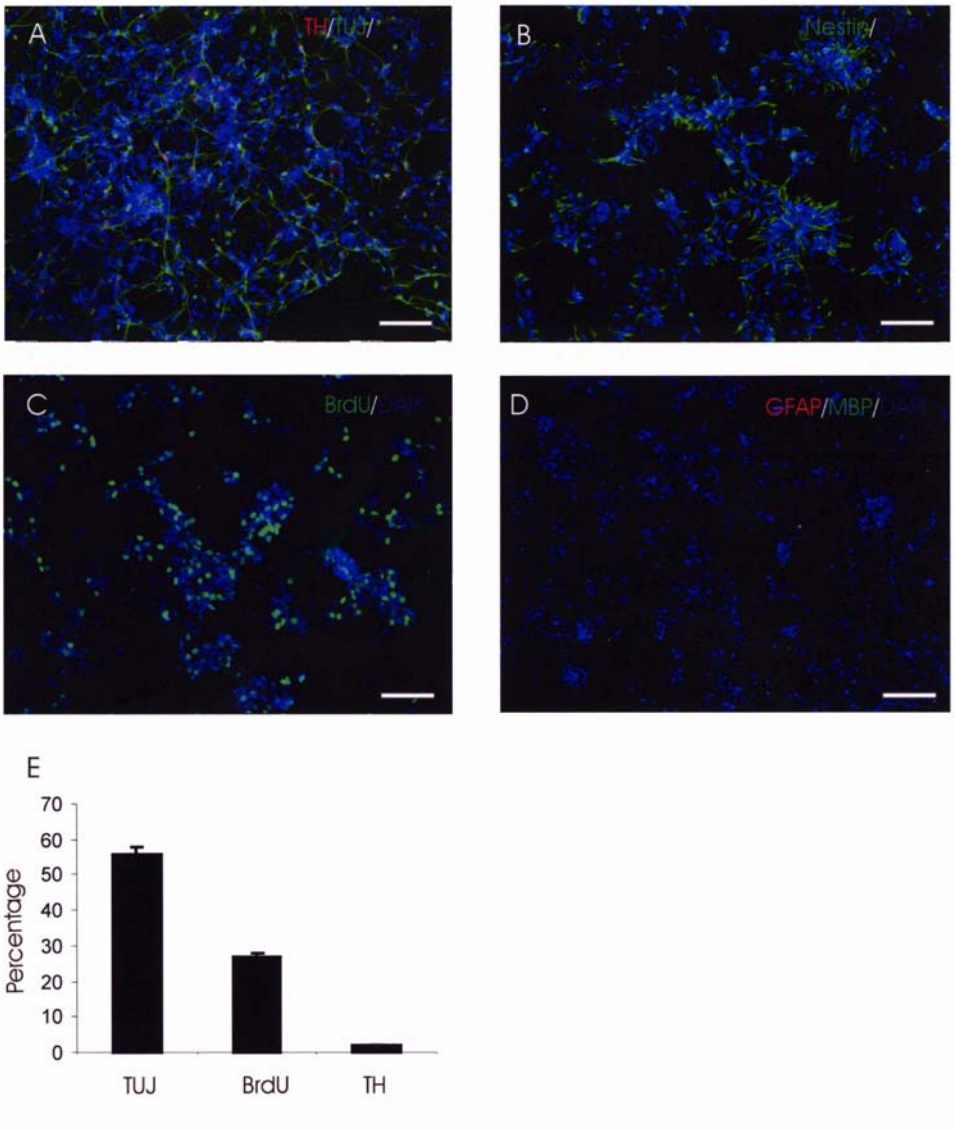

A
TH/TUJ/
B
Nestin/
C
BrdU/
D
GFAP/MBP/
E
Percentage
70
60
50
40
30
20
10
0
TUJ
BrdU
TH

Figure 9.4:

Photomicrographs showing representative cultures of E12 rat VM grown for 1 DIV, immunocytochemically stained for TH (green) and DAPI (blue). (A) shows a control culture, (B) shows a culture that received 10ng/ml GDF-5 at the time of plating. (C) Graphical representation showing the average number of TH-positive neurons per field in control and GDF-5 treated cultures. Data represent mean ± SEM taken from three independent experiments.

*P<0.05 compared to control cultures

Scale bar = 100μm

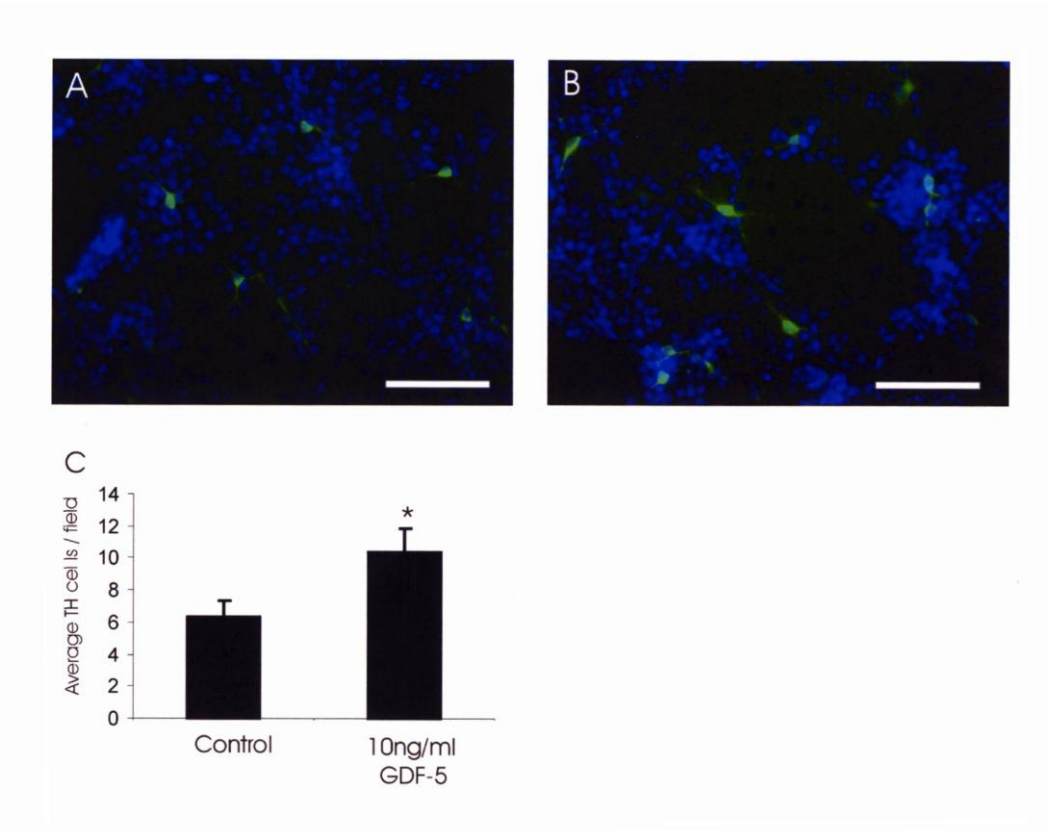


Figure 9.5:

Graphical representations showing (A) the average numbers of apoptotic cells per field and (B) the average number of total cells per field in control and GDF-5 treated cultures after 1 DIV. Data represent mean ± SEM taken from three independent experiments.

N.S. no significant difference between control and GDF-5 treated cultures

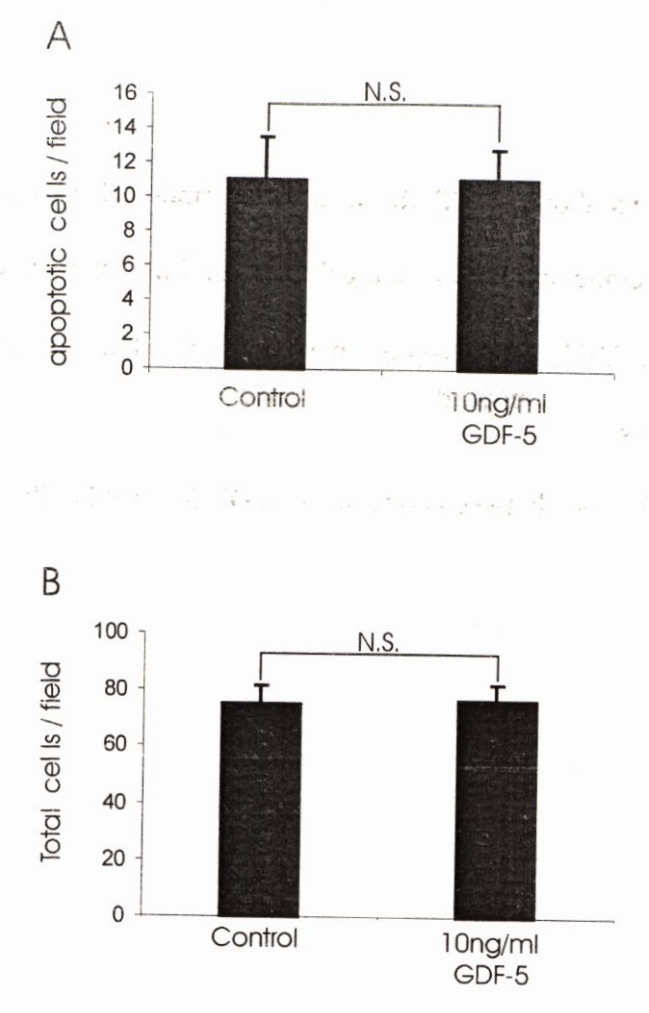


Figure 9.6:

Photomicrographs showing representative cultures of E12 rat VM grown for 1 DIV, immunocytochemically stained for PCNA (red), TH (green) and DAPI (blue). (A) shows a control culture, (B) shows a culture that received 10ng/ml GDF-5 at the time of plating. (C) Graphical representation showing the average number of BrdU-positive nuclei per field in control and GDF-5 treated cultures. Data represent mean ± SEM taken from two independent experiments.

N.S. no significant difference between control and GDF-5 treated cultures

Scale bar = 20μm

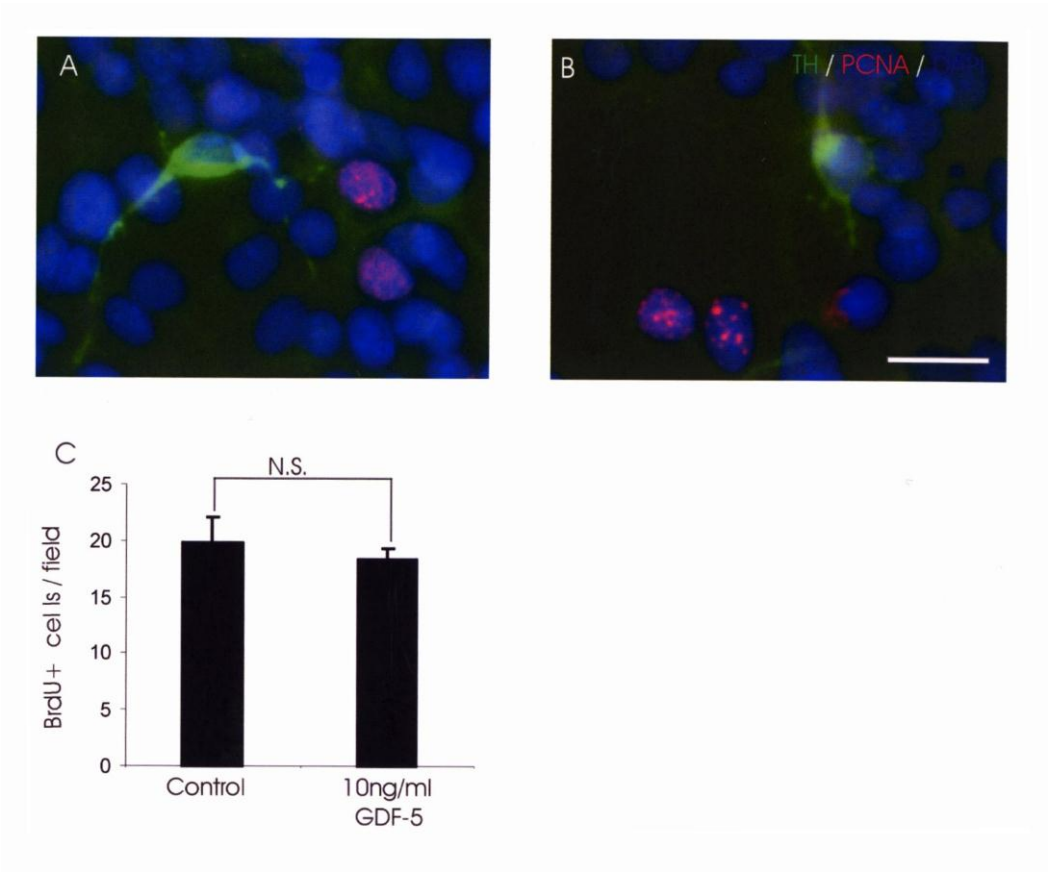
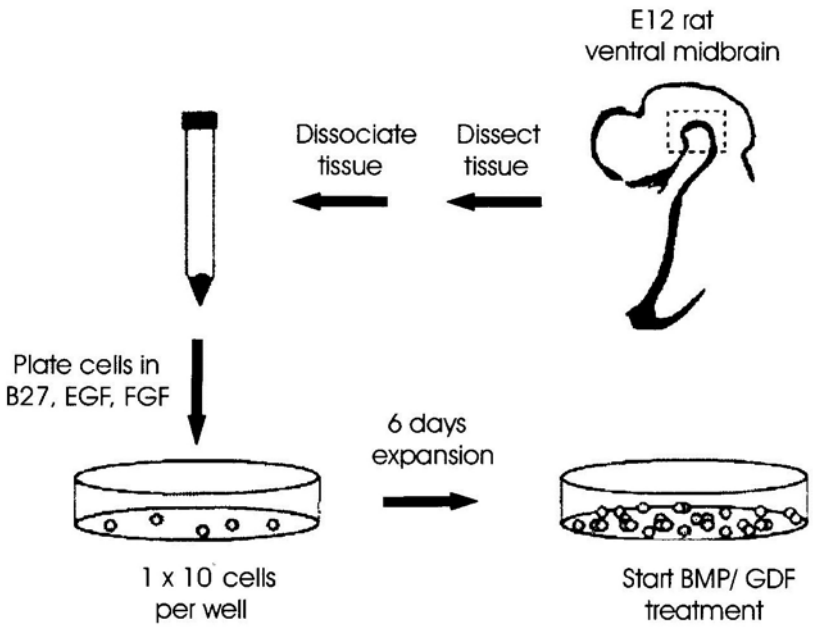


Figure 9.7:

Diagram showing the experimental protocol used to examine gene expression in expanded neural progenitor cells from the E12 VM. 1x 10^4 cells were plated in complete medium (see appendix A) supplemented with 2% B27, 20ng/ml EGF and 20ng/ml FGF and expanded for 6 DIV. After 6 DIV GDF-5, BMP-2 and combinations of both were added to the culture medium. 24hours later medium was removed and RNA extracted for analysis by RT-PCR.



Adapted from Panchision et al. 2001

Figure 9.8:

Phase contrast photomicrographs of E12 NPCs grown in medium containing (A) 20ng/ml EGF and 20ng/ml FGF, (B) 20ng/ml EGF and (C) 20ng/ml FGF, for 6 DIV.

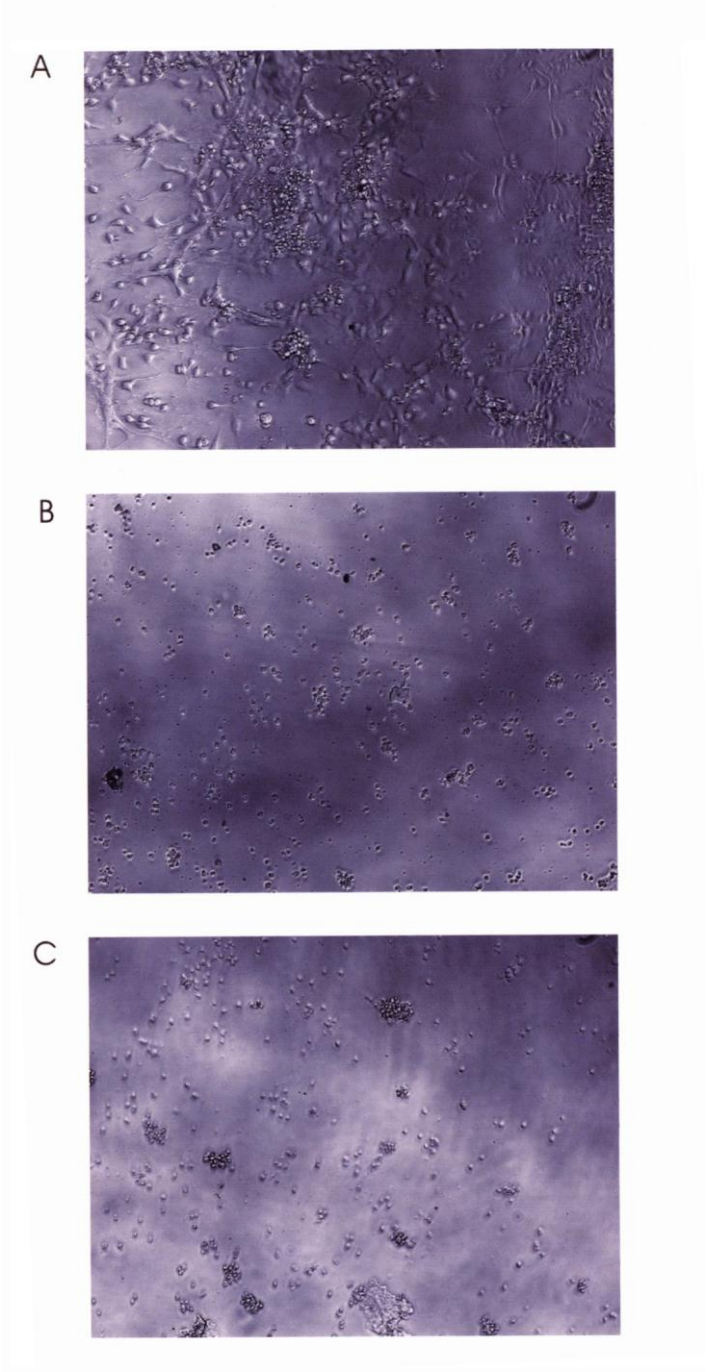


Figure 9.9:

RT-PCR analysis of (A) BMPR-Ia, (B) BMPR-IB, (C) Ror2, (D) Nurr 1, (E) TH, (F) Msx1, (G) positive control for Msx-1 amplified from cDNA prepared from the adult rat brain and (H) 18s RNA gene expression in expanded neural progenitors from the E12 rat VM following the treatments shown.

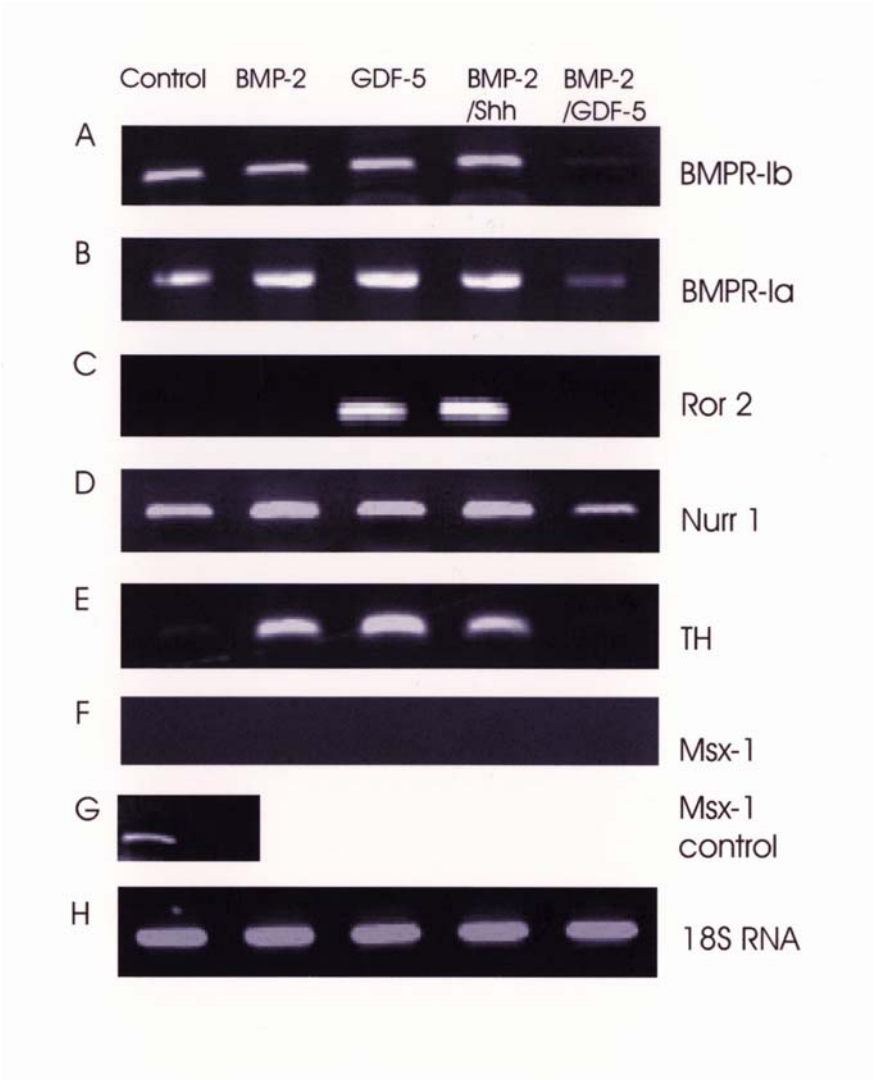


Figure 9.10:

Photomicrographs showing representative cultures of expanded neural progenitors prepared form the E12 rat VM expanded for 6 DIV and allowed to differentiate for a further 6 DIV in (A) control medium and (B) in medium containing 50ng/ml GDF-5. Cultures were immunocytochemically stained for TH (red), TUJ (green) and DAPI (blue).

Scale bar = 100μm

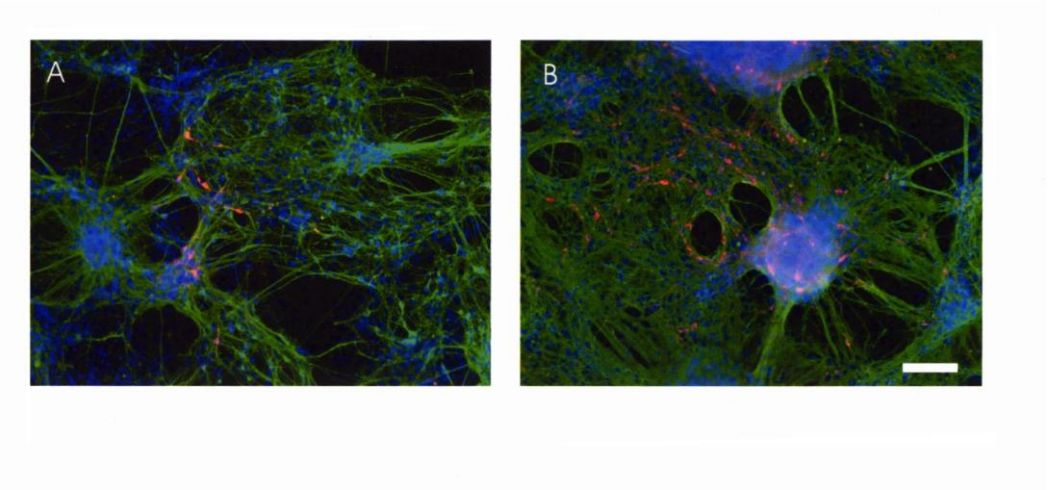
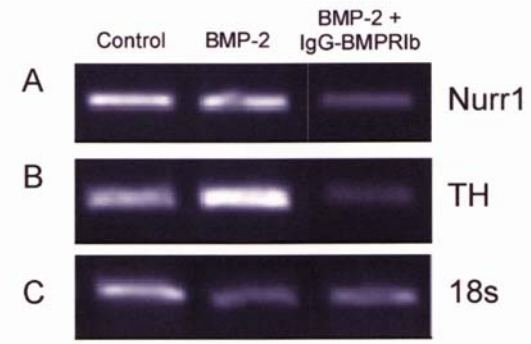
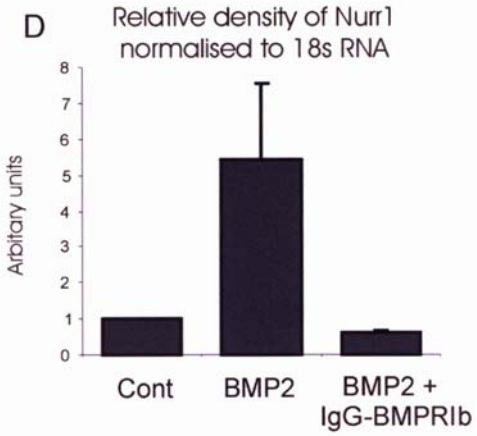
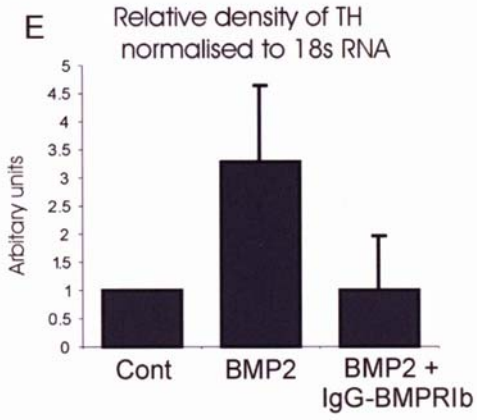


Figure 9.11:

RT-PCR analysis of (A) nurr1 (B) TH and (C) 18s RNA gene expression in expanded neural progenitors from the E12 rat VM following the treatments shown. Graphical representations of (D) nurr1 levels and (E) TH levels in control cultures compared to those in BMP-2 and BMP-2/IgG-BMPR-Ib cultures. Data was generated by densiometric analysis comparing nurr1 and TH levels normalised to those of 18s RNA.


Control
BMP-2
BMP-2 +
IgG-BMPRIb
A
Nurr1
B
TH
C
18s


D
Relative density of Nurr1
normalised to 18s RNA
Arbitary units
0
1
2
3
4
5
6
7
8
Cont
BMP2
BMP2 +
IgG-BMPRIb


E
Relative density of TH
normalised to 18s RNA
Arbitary units
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Cont
BMP2
BMP2 +
IgG-BMPRIb

10.0 FINAL DISCUSSION

Experimental evidence has resulted in GDF-5 being proposed as a candidate neurotrophin for the treatment of PD. Based on published studies, GDF-5 improves the survival of embryonic dopaminergic neurons grafted to the striatum in a rat model of PD (Sullivan et al. 1998). Furthermore GDF-5 protects adult nigrostriatal dopaminergic neurons when directly administered to the striatum or SN in a rat model of PD (Sullivan et al. 1997, 1999; Hurley et al. 2004). If GDF-5 is to be used in a clinical setting for the treatment of PD, many questions must be answered regarding it roles and expression pattern during brain development and in particular its roles in dopaminergic neuron development.

Prior to the beginning of this thesis, it was not known if GDF-5 was expressed in the developing or adult nigrostriatal system. These studies have shown that GDF-5 is associated with both the developing and adult nigrostriatal system (O'Keeffe et al. 2004a), which suggests that it plays a role in dopaminergic neuron development and maintenance in the adult brain. Furthermore these studies have documented at least some of the roles that GDF-5 plays in dopaminergic development. GDF-5 increases the numbers of dopaminergic neurons in cultures of E14 rat VM. This increase in dopaminergic neuron numbers is achieved by increasing the conversion of Nurr-1-positive, TH-negative dopaminergic precursor cells to Nurr-1-positive TH-positive dopaminergic neurons. Furthermore GDF-5 increases the branching and neurite length of these neurons, suggesting that it plays a role in their morphological development of these neurons (O'Keeffe et al. 2004b).

It will be important to determine the precise mechanisms involved in these processes. The activation of intracellular smad proteins by GDF-5 implies that the effects of GDF-5 treatment on dopaminergic neurons are (at least in part) mediated by

smad signaling. The presence of Ror2, which is a novel receptor for GDF-5, in the VM at E14 makes it possible that GDF-5 can use alternative signaling pathways other than the smad pathway. Some pilot data has also shown that GDF-5 can activate Erk-MAPK kinase in cultures of E14 rat VM (O'Keeffe et al. unpublished observations). As this pathway has been shown to inhibit smad signaling (Kretzchmar et al. 1997), it will be important to determine the relationship between these signaling pathways.

As well as the application of this research to a practical problem, that is generating dopaminergic neurons from NPCs, it also raises a fascinating point regarding how cell fate in specified in the nervous system. These findings show that BMP-2, which to date has been shown to induce a dorsal fate in proliferating ventral cells, can also induce a ventral cell fate, in the presence of EGF. It will be important to fully explore the relationship between the EGF signalling pathway and the signalling pathways activated by BMPs or GDFs, in order to fully understand how the same ligand can induce a dorsal or ventral cell fate in the neural tube. Although BMPs are classically known as factors that induce a dorsal fate in NPCs during neural tube development, it is the opinion of this author that under certain circumstances that they may be involved in the induction of a ventral cell fate in the developing of the neural tube. The species differences observed in these studies regarding the expression of BMPR-Ib, which is being expressed in the ventral midbrain in the rat but not in the mouse, implies that care must be taken when interpreting the findings of mouse genetic studies examining the roles of these receptors during CNS development (for example see Panchision et al. 2002).

What is clear from these studies is that the effects of GDF-5 on dopaminergic neurons make it a very suitable candidate neurotrophin to use as an adjunct to neural transplantation of embryonic dopaminergic neurons in the treatment of PD. GDF-5 is

perhaps a more suitable neurotrophin than GDNF for use in supporting grafted embryonic rat dopaminergic neurons, as the expression of GDF-5 in the VM is maximal on E14 the day that the neurons are routinely harvested for transplantation studies (O'Keeffe et al. 2004a), unlike that of GDNF whose expression increases in the postnatal period (O'Keeffe et al. unpublished observations). Furthermore the increase in dopaminergic neuron numbers as well as the improvements seen in the morphological development of these neurons make suggest that GDF-5 may improve integration of the grafted dopaminergic neurons into the host brain by inducing axonal outgrowth from the transplanted cells and the establishment of functional connections with the host brain (for a summary of roles of GDF-5 during rat VM development see Figure 10.1).

Neurotransplantation for the treatment of PD is limited, due to ethical and practical concerns regarding the use of human embryonic tissue. One of the most limiting problems is the requirement for 6-8 embryos to treat a single patient. The widespread adoption of clinical neurotransplantation for the treatment of PD is unlikely unless alternative sources of dopaminergic neurons are found. As NPCs can be grown efficiently in large numbers and differentiated into the required neuronal phenotype, they are an ideal source of cells for use in transplantation approaches to neurodegenerative disease.

One of the studies presented here show that the gestational age of the donor tissue used to generate the NPCs is a key factor in determining the cell types generated. It was found that NPCs expanded from the rat E12 VM for 6 DIV and differentiated for 6 DIV could generate dopaminergic neurons, whereas those from later ages could not. However, the ability to generate dopaminergic neurons was only observable on the first passage and subsequently lost with increasing time in culture. These studies have shown that GDF-5 (and BMP-2) induce the expression of nurr-1 and TH in proliferating

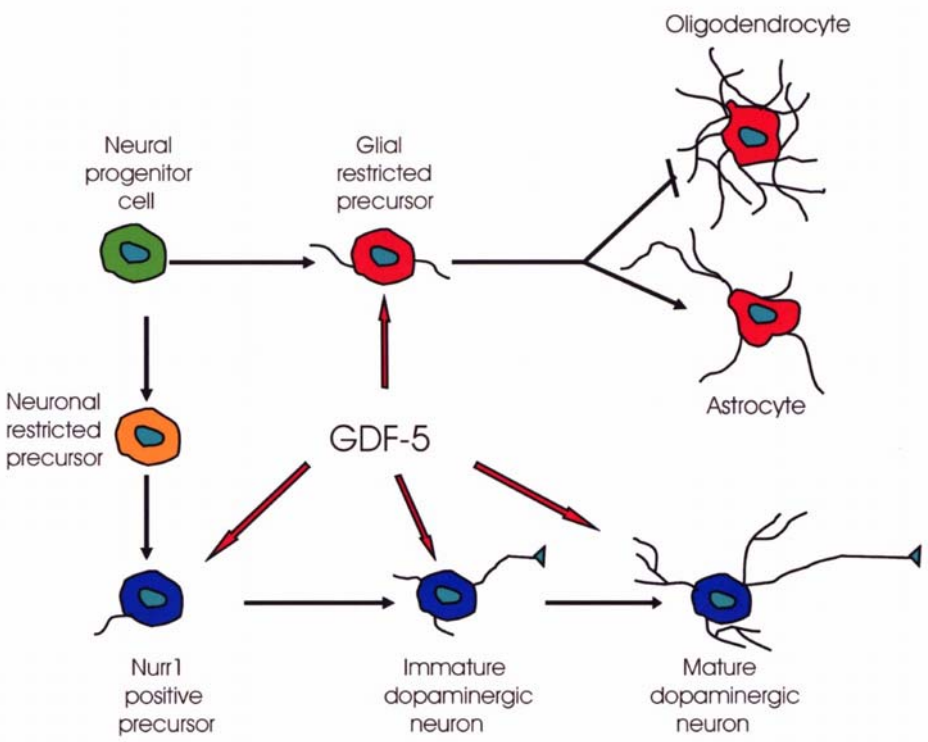
NPCs from the E12 rat VM. This makes these factors candidates for further studies aimed at examining whether they could be used to efficiently generate dopaminergic neurons from NPCs for use in transplantation approaches to PD.

Transplantation of expanded NPCs (or cells derived from these) is the most hopeful course for generating dopaminergic neurons for transplantation approaches to PD. Although NPCs can be isolated from a variety of brain regions, it is the opinion of this author that the NPCs should be isolated from regions of the embryonic brain that give rise to the types of neurons which are affected by the disease process. For example, for transplantation approaches to PD, NPCs should be isolated from the embryonic VM, while for transplantation approaches to Huntingtons disease, they should be isolated from the embryonic striatum. At the present time not enough is understood about NPCs (or other stem cell sources) to begin clinical transplantation. Centres and clinical trials that offer transplants of stem cells to patients in the current day are extremely damaging to the field of transplantation as these simply are not effective. Not enough is known about the basic biology of the stem cell and how it differentiates to generate a mature neuronal cell. For example, although much is know about how VM dopaminergic neurons develop *in vivo*, much more remains to elucidated. The generation of mature fully functional dopaminergic neurons during normal VM development is likely to require and be controlled by a multitude of different factors and signaling pathways. Whether we can ever simulate such a complex process in a culture dish to differentiate NPCs to mature, fully functional dopaminergic neurons remains to be seen. This problem is highlighted by a recent report presented at a stem cell meeting in Boston. Dopaminergic neurons were efficiently generated from human ES cells *in vitro*, but when these cells were transplanted to the striatum in animal models of PD, none of them survived. Much more research needs to be carried out to determine how these neurons develop, mature, become electrically active and maintain a dopaminergic phenotype.

Only when scientists can show that dopaminergic neurons generated from NPCs or other stem cell sources have all the properties of mature fully functional midbrain dopaminergic neurons isolated directly from the midbrain should such cells be considered for transplantation. Until that point is reached, medical, pharmacological and surgical treatment for PD should be pursued.

Figure 10.1:

Schematic representation showing functions of GDF-5 during rate VM development. GDF-5 increases the conversion of Nurr-1-positive, TH-negative precursors to Nurr-1-positive, TH-positive dopaminergic neurons. GDF-5 also improves the morphological development of dopaminergic neurons. The results of the present study taken with those of a previous study (Brederlau et a. 2002) show that GDF-5 promotes differentiation of GRPs to an astroglial fate at the expense of an oligodendroglial fate.



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12.0 APPENDICES

12.1 APPENDIX A (GENERAL APPENDIX)

Antibodies used in western blotting

Antibody	*Working dilution*
GDF-5 (P3/66) (Monoclonal; Biopharm)	1:1,000
β-III tubulin (Monoclonal; Promega)	1:50,000
GFAP (Monoclonal; Sigma)	1:1,000
GFAP (Polyclonal-rb*; DAKO)	1:1,000
TH (Polyclonal-rb; Chemicon)	1:1,000
Actin (Polyclonal-gt; Santa-cruz)	1:10,000

(* -rb = polyclonal antibody raised in rabbit, -gt = polyclonal antibody raised in goat).

10% ($^{w}/_{v}$) ammonium persulphate (APS)

0.1g of APS was dissolved in 1ml of distilled water. Fresh APS was made up for each new western blot.

Biotinylated molecular weight markers (Amersham):

25µl of the molecular weight markers were mixed with 225µl of the sample bufferfor the molecular weight markers (see below). 10µl aliquots were stored at –20°C. 10µl molecular weight markers were boiled for 5 mins before being loaded onto the gel.

1% ($^{w}/_{v}$) Bromophenol blue

0.1g bromophenol blue (Sigma) was dissolved in 10ml of distilled water. Solution was stored at room temperature.

Coomassie blue staining

Coomassie dye (Sigma)	2.5g
Ethanol (AGB)	500ml
Glacial acetic acid	100ml
Distilled water	200ml

Destaining solution:

Ethanol (AGB)	250ml
Glacial acetic acid	70ml
Distilled water	680ml

The gel was incubated in staining solution overnight at 4°C. Excessive staining was removed by incubating the stained gel in destaining solution with gentle agitation until an appropriate contrast was reached.

Gel recipes

Percentage Gels	*5%*	*10%*	*12%*
Distilled water	5.7ml	4.1ml	3.4ml
*Acrylamide	1.7ml	3.3ml	4.0ml
*0.5M Tris pH 6.8	2.5ml	2.5ml	2.5ml
*1.5M Tris pH8.8	2.5ml	2.5ml	2.5ml
10% SDS (Sigma)	100µl	100µl	100µl
10% APS (Sigma)	50µl	50µl	50µl
TEMED (Sigma)	10µl	10µl	10µl

(*Bio-Rad)

Homogenate buffer for cell/tissue lysis

1M Tris pH7.4 (Bio-rad)	1ml
NaCl (Sigma)	0.18g
Glycerol (Sigma)	2ml
Triton X-100 (Sigma)	200µl
0.5M EDTA (Sigma)	40µl
PMSF	100µg/ml
Bacitracin	1µg/ml
Pepstatin A	1µg/ml
Aprotinin	1µg/ml
Leupeptin	1µg/ml
Antipain	1µg/ml
Distilled water	Made up to 20ml

10x PBS (100mM)

Sodium chloride	33.64g
Di-sodium hydrogen orthophosphate	11.86g
Di-sodium hydrogen orthophosphate di-hydrate	46.0g
Distilled water	Made up to 1 liter

To make a 1x PBS solution (10mM), 100ml was added to 900ml of distilled water. Solution was stored at room temperature.

PBST (1x)

1 liter of 10mM PBS + 1ml of tween 20 (Sigma). Solution was stored at room temperature.

PBS-Tx (1x)

1 liter of 10mM PBS + 1ml of 20% Triton-X 100 (Sigma). Solution was stored at room temperature.

Ponceau S staining of the PVDF membrane

10 x Ponceau S staining solution:

Ponceau S (Sigma)	2g
Acetic Acid	50ml
Distilled water	50ml

PVDF membranes were stained by placing them in 1 x Ponceau S solution (1ml 10x stock + 9ml distilled water) for 10 mins. Following this blots were washed in several changes of distilled water to remove the Ponceau S staining.

Running buffer (5x)

Tris-base (Sigma)	15.15g
Glycine (Sigma)	72.50g
SDS (Sigma)	5g
Distilled Water	Made up to 1 liter

A 1x solution was mage by diluting the 5x stock with distilled water (100ml 5x stock + 400ml distilled water)

Sample buffer for cell/tissue samples

Urea (Sigma)	8.4g
sodium-phosphate (Sigma)	0.02g
SDS (Sigma)	0.2g
1% bromophenol blue (Sigma)	200µl
dithiothreitol (Sigma)	0.31g

Distilled water	Made up to 20ml

Sample buffer for molecular weight markers

0.5M Tris pH 6.8 (Bio-rad)	1ml
Glycerol (Sigma)	0.8ml
10% SDS (Sigma)	1.6ml
β-metacarpoethanol(Sigma)	0.4ml
1%($^{w}/_{v}$) bromophenol blue(Sigma)	0.2ml
Distilled water	4ml

10% ($^{w}/_{v}$) SDS solution:

10g of SDS (Sigma) was dissolved in 100ml of distilled water. Solution was stored at room temperature.

Transfer buffer (1x)

Tris-base (Sigma)	5.82g
Glycine (Sigma)	2.93g
Methanol (Sigma)	50ml
Distilled water	Made up to 1 liter

12.2 APPENDIX B (CELL CULTURE)

12.2.1 Poly-D-lysine coating of culture dishes

A 0.1mg/ml solution of poly-d-lysine (Sigma) was made by dissolving 5 mg of poly-d-lysine in 50 ml of distilled water. This solution was applied to the 13mm coverslips or the wells of the 3cm 4 well dishes. It was left covering the surfaces for 20 mins, before being removed. The coverslips and dishes were washed (3 x 10 mins) in distilled water. Coverslips/culture dishes were allowed to dry at 37°C for one h before use. Any unused poly-d-lysine solution was aliquoted into 5 ml aliquoted and stored at –20°C.

12.2.2 Culture media

GDF5-CHO cell line serum medium

MEMα	450ml
FCS	50ml
L-glutamine	2mM
Methotrexate	400nM
Penicillin	100U/ml
Streptomycin	100μg/ml

GDF5-CHO cell line serum-free medium

DMEM/Ham's F12 (1:1) with 15mM HEPES and sodium bicarbonate (Sigma)	500ml
Aprotinin	10 KIU
Sodium butyrate	1mM
Sodium selenate	6.7ng/ml
Transferrin	5.5μg/ml

Ethanolamine	2μg/ml
Insulin	9μg/ml
Penicillin	100U/ml
Streptomycin	100μg/ml

Complete medium for cultures of rat neural cells

DMEM/Ham's F12 (1:1) with 15mM HEPES	
and sodium bicarbonate (Sigma)	500ml
L-glutamine (Sigma; 100x)	5ml
Pen/Strep/Fungizone (Sigma; 100x)	5ml
*D-glucose (Sigma)	3g

(* 3g of D-Glucose were dissolved in 10 ml of DMEM/F12 and sterile filtered into the remaining 490ml of DMEM/F12)

Complete medium was stored in the original bottle, wrapped in tin foil and stored at 4°C.

Plating medium for cultures of E14 rat VM

Complete medium	9.7ml
B27 (Gibco; 50x)	200μl
Foetal calf serum (Sigma; FCS)	100μl

Serum-free medium for cultures of E12 and E14 rat VM

Complete medium	9.46ml
B27 (Gibco; 50x)	200μl
BSA (Sigma; 7.5%)	333μl

Expansion medium for neural progenitor cell culture

Complete medium	9.9ml
N2 (Gibco; 100x)	100µl
EGF (20µg/ml stock; Sigma)	10µl
FGF-2 (20µg/ml stock; RnD Systems)	10µl

Differentiation medium for neural progenitor cell culture

Complete medium	9.9ml
N2 (Gibco; 100x)	100µl
FCS (Sigma)	100µl

DAPI:

Stock solution of DAPI was prepared by dissolving 10mg of DAPI powder (Sigma) in 10mM PBS. 500 µl aliquots were stored in the dark at -20°C. A working solution was prepared by diluting the stock 1:100 in 10mM PBS to give a final working concentration of 10µg/ml.

12.2.3 Buffers for reconstitution of growth factors

Sodium citrate buffer:

Sodium citrate (Sigma)	0.02g
Sodium chloride (Sigma)	0.09g
Distilled water	Made up to 10ml

100mM DTT stock:

0.31g of DTT (Sigma) was dissolved in 20ml of 10mM PBS. The solution was sterile filtered and stored at 4°C.

50 mg/ml Heparin:

50 mg of heparin (Sigma) was dissolved in 1 ml of 10mM PBS. The solution was sterile filtered and stored at 4°C.

Reconstitution of growth factors:

GDF-5 (Biopharm):

100μg of recombinant human GDF-5 was reconstituted using 0.2 μM filtered sterile sodium-citrate buffer to give a stock solution of 100μg/ml. 20μl aliquots were prepared and stored at

–20°C.

EGF (Sigma):

25μg of EGF was reconstituted using 0.2 μM filtered sterile 10mM acetic acid containing 0.1% BSA (Sigma) to give a stock solution of 20μg/ml. 20μl aliquots were prepared and stored at –20°C.

FGF-2 (R &D systems):

25μg of FGF-2 was reconstituted using 0.2 μM filtered sterile 10mM PBS containing 0.1% BSA (Sigma), 1mM DTT (Sigma) and heparin (Sigma) at a final concentration of 5μg/ml, to give a stock solution of 20μg/ml. 20μl aliquots were prepared and stored at –20°C.

Shh (R &D systems):

25μg of recombinant human Shh was reconstituted using 0.2 μM filtered sterile 10mM PBS containing 0.1% BSA (Sigma) to give a stock solution of 50μg/ml. 20μl aliquots were prepared and stored at –20°C.

FGF-2 (R &D systems):

25μg of recombinant mouse FGF-2 was reconstituted using 0.2 μM filtered sterile 10mM PBS containing 0.1% BSA (Sigma) to give a stock solution of 50μg/ml. 20μl aliquots were prepared and stored at –20°C.

BMP-2:

25μg of recombinant human BMP-2 was reconstituted using 0.2 μM filtered sterile 4mM HCl containing 0.1% BSA (Sigma) to give a stock solution of 10μg/ml. 20μl aliquots were prepared and stored at –20°C.

Blocking solution for immunocyotchemistry:

Normal horse serum (Sigma)	1ml
20% Triton X-100	200μl
10mM PBS pH 7.4	18.8ml

Solution was stored at 4°C for up 7 days before fresh blocking solution was prepared.

Primary antibodies used in immunocytochemistry:

Antibody:	*Working dilution for immunocyotchemistry:*
GDF-5/MP-52 (Monoclonal; Biopharm)	1:50
β-III tubulin (Monoclonal; Promega)	1:200
MAP-2_{ab} (Monoclonal; Sigma)	1:100
GFAP (Monoclonal; Sigma)	1:200
GFAP (Polyclonal-rb*; DAKO)	1:400
PCNA (Monoclonal; Sigma)	1:100
Nestin (Monoclonal; Chemicon)	1:200
Nurr1 (Polyclonal-gt*; Santa-cruz)	1:1000
Nurr1 (Polyclonal-rb; Chemicon)	1:1000

TH (Polyclonal-rb; Chemicon)	1:200
BMPR-II (Polyclonal-gt*; Santa-cruz)	1:10
BMPR-Ia (Polyclonal-gt; Santa-cruz)	1:10
BMPR-Ib (Polyclonal-gt; Santa-cruz)	1:10

(* -rb = polyclonal antibody raised in rabbit, -gt = polyclonal antibody raised in goat).

12.3 APPENDIX C (CHAPTER 6)

12.3.1 COUNTS OF TH-POSITIVE CELLS IN E14VM CULTURES AFTER TREATMENT WITH GDF-5 FOR 6 DIV

	Control		1ng/ml GDF-5		10ng/ml GDF-5	
c.slip no. / field no.	TH (+) cells/field	Average	TH (+) cells/field	Average	TH (+) cells/field	Average
1.1	1		14		20	
1.2	10		17		9	
1.3	11		20		12	
1.4	7		12		11	
1.5	4		14		17	
1.6	6		13		15	
1.7	3		21		18	
1.8	13	6.9	19	16.3	14	15
2.1	7		13		10	
2.2	8		13		13	
2.3	9		14		15	
2.4	5		11		19	
2.5	8		30		14	
2.6	4		9		13	
2.7	7		19		11	
2.8	2	6.3	10	14.8	13	14
3.1	0		11		17	
3.2	5		15		15	
3.3	5		7		14	
3.4	10		13		16	
3.5	6		27		15	
3.6	2		21		24	
3.7	1		18		18	
3.8	6	4.4	17	16.2	7	15.8
4.1	6		16		9	
4.2	4		17		22	
4.3	6		15		17	
4.4	7		19		11	
4.5	5		18		21	
4.6	5		25		11	
4.7	4		17		12	
4.8	6	5.4	9	17	13	15
5.1	3		12		10	
5.2	6		16		19	
5.3	6		21		16	
5.4	2		16		11	
5.5	3		12		20	
5.6	8		13		11	
5.7	6		10		10	
5.8	6	5	15	14.4	12	13.7
6.1	2		16		15	
6.2	4		21		24	
6.3	7		19		21	
6.4	4		16		14	
6.5	5		15		14	
6.6	3		14		18	
6.7	6		14		13	
6.8	4	4.4	12	15.9	14	16.7
7.1	3		7		8	
7.2	2		9		6	
7.3	2		18		11	
7.4	8		11		11	
7.5	3		21		6	
7.6	5		14		8	
7.7	2		10		11	
7.8	4	3.6	12	12.8	3	8
8.1	2		9		5	
8.2	2		6		3	
8.3	3		17		10	
8.4	8		19		10	
8.5	3		18		5	
8.6	2		14		6	
8.7	4		14		5	
8.8	3	3.4	10	13.4	7	6.4
9.1	2		9		6	
9.2	3		7		8	
9.3	4		8		6	
9.4	5		14		7	
9.5	2		14		10	
9.6	4		14		11	
9.7	3		7		9	
9.8	3	3.3	10	10.4	6	7.9
10.1	3		9		7	
10.2	2		14		5	
10.3	5		12		11	
10.4	8		13		9	
10.5	4		15		5	
10.6	3		20		6	
10.7	1		16		11	
10.8	1	3.4	8	13.4	8	7.8
MEAN		**4.6**		**14.4**		**11.9**
SD		**1.3**		**2.0**		**3.9**
SE		**0.4**		**0.6**		**1.2**

12.3.2 COUNTS OF GFAP-POSITIVE CELLS AFTER TREATMENT WITH GDF-5 FOR 6 DIV

	Control		1ng/ml GDF-5		10ng/ml GDF-5	
c.slip no. / field no.	GFAP (+) cells/field	Average	GFAP (+) cells/field	Average	GFAP (+) cells/field	Average
1.1	8		16		24	
1.2	5		13		18	
1.3	8		18		56	
1.4	9	7.5	11	14.5	34	33
2.1	4		14		73	
2.2	9		10		36	
2.3	6		15		62	
2.4	7	6.5	17	14	41	53
3.1	8		17		74	
3.2	9		13		71	
3.3	8		18		38	
3.4	5	7.5	25	18.3	29	53
4.1	10		12		61	
4.2	6		18		42	
4.3	10		16		43	
4.4	8	8.5	25	17.8	74	55
5.1	10		32		41	
5.2	8		8		42	
5.3	11		15		50	
5.4	7	9	14	17.3	29	40.5
6.1	7		20		32	
6.2	6		13		61	
6.3	4		14		57	
6.4	6	5.8	10	14.3	66	54
7.1	4		10		63	
7.2	4		13		20	
7.3	5		26		47	
7.4	3	4	20	17.3	50	45
8.1	5		22		48	
8.2	6		18		26	
8.3	6		9		63	
8.4	8	6.3	13	15.5	27	41
9.1	9		33		30	
9.2	11		5		42	
9.3	15		16		40	
9.4	11	11.5	13	16.8	30	35.5
10.1	10		13		33	
10.2	15		21		37	
10.3	10		21		45	
10.4	6	10.3	9	16	41	39
11.1	18		36		43	
11.2	15		34		63	
11.3	11		18		44	

11.4	8	13	13	25.3	44	48.5
12.1	10		41		32	
12.2	4		27		39	
12.3	11		26		47	
12.4	6	7.8	38	33	69	46.8
MEAN	**8.1**			**18.3**		**45.4**
SD	**2.5**			**5.5**		**7.6**
SE	**0.7**			**1.6**		**2.2**

12.3.3 COUNTS OF TH-POSITIVE CELLS AFTER TREATMENT WITH GDF-5 FOR 24 HS AT 1 OR 6 DIV

	1DIV				**7DIV**			
	Control	**10ng/ml GDF-5**			**Control**	**10ng/ml GDF-5**		
c.slip no. / field no.	**TH (+) cells/field**	**TH (+) cells/field**	**Average/field**	**Average/field**	**TH (+) cells/field**	**TH (+) cells/field**	**Average/field**	**Average/field**
1.1	16	18			81	88		
1.2	31	23			70	86		
1.3	23	21			76	77		
1.4	16	21	21.5	20.75	44	76	67.75	81.75
2.1	26	20			93	91		
2.2	13	33			103	74		
2.3	22	33			64	82		
2.4	12	46	18.25	33	40	79	75	81.5
3.1	29	18			83	81		
3.2	9	19			50	89		
3.3	60	19			63	107		
3.4	34	30	33	21.5	68	67	66	86
4.1	24	28			70	93		
4.2	26	41			56	64		
4.3	11	27			71	96		
4.4	31	22	23	29.5	62	62	64.75	78.75
5.1	25	29			71	93		
5.2	29	11			73	82		
5.3	33	22			70	97		
5.4	33	27	30	22.25	60	71	68.5	85.75
6.1	4	32			54	73		
6.2	18	14			57	88		
6.3	22	22			42	83		
6.4	50	17	23.5	21.25	59	122	53	91.5
MEAN			**24.9**	**24.7**			**65.9**	**84.2**
SD			**5.5**	**5.2**			**7.2**	**4.5**
SE			**3.9**	**3.7**			**5.1**	**3.2**

12.3.4 MEASUREMENT OF NEURITE LENGTH

Control **Number of intercepts at different levels of neurite field** **Neurite length in µm at different levels of neurite field**

	c slip no.	**Primary**	**Sec**	**Ter**	**Quat**	**Total**	**Primary**	**Sec**	**Ter**	**Quat**	**Total**
Neuron 1	1	5	0	0	0	5	57.1	0.0	0.0	0.0	57.1
Neuron 2	1	5	4	0	0	9	57.1	45.7	0.0	0.0	102.8
Neuron 3	1	6	6	0	0	12	68.5	68.5	0.0	0.0	137.1
Neuron 4	1	8	0	0	0	8	91.4	0.0	0.0	0.0	91.4
Neuron 5	2	2	7	3	0	12	22.8	80.0	34.3	0.0	137.1
Neuron 6	2	6	0	0	0	6	68.5	0.0	0.0	0.0	68.5
Neuron 7	2	4	2	0	0	6	45.7	22.8	0.0	0.0	68.5
Neuron 8	3	2	6	1	0	9	22.8	68.5	11.4	0.0	102.8
Neuron 9	3	4	1	0	0	5	45.7	11.4	0.0	0.0	57.1
Neuron 10	3	8	2	0	0	10	91.4	22.8	0.0	0.0	114.2
Neuron 11	3	11	0	0	0	11	125.7	0.0	0.0	0.0	125.7
Neuron 12	4	4	7	0	0	11	45.7	80.0	0.0	0.0	125.7
Neuron 13	4	5	4	0	0	9	57.1	45.7	0.0	0.0	102.8
Neuron 14	4	13	1	0	0	14	148.5	11.4	0.0	0.0	159.9
Neuron 15	4	4	2	0	0	6	45.7	22.8	0.0	0.0	68.5
Neuron 16	5	10	2	0	0	12	114.2	22.8	0.0	0.0	137.1
Neuron 17	5	8	0	0	0	8	91.4	0.0	0.0	0.0	91.4
Neuron 18	5	3	7	0	0	10	34.3	80.0	0.0	0.0	114.2
Neuron 19	5	11	0	0	0	11	125.7	0.0	0.0	0.0	125.7
Neuron 20	5	1	4	0	0	5	11.4	45.7	0.0	0.0	57.1
Neuron 21	6	20	0	0	0	20	228.5	0.0	0.0	0.0	228.5
Neuron 22	6	3	4	0	0	7	34.3	45.7	0.0	0.0	80.0
Neuron 23	6	8	0	0	0	8	91.4	0.0	0.0	0.0	91.4
Neuron 24	6	12	0	0	0	12	137.1	0.0	0.0	0.0	137.1
Neuron 25	6	4	0	0	0	4	45.7	0.0	0.0	0.0	45.7
Neuron 26	7	8	5	0	0	13	91.4	57.1	0.0	0.0	148.5
Neuron 27	8	8	0	0	0	8	91.4	0.0	0.0	0.0	91.4
Neuron 28	8	6	0	0	0	6	68.5	0.0	0.0	0.0	68.5
Neuron 29	9	5	4	0	0	9	57.1	45.7	0.0	0.0	102.8
Neuron 30	9	4	0	0	0	4	45.7	0.0	0.0	0.0	45.7
Neuron 31	9	9	0	0	0	9	102.8	0.0	0.0	0.0	102.8
Neuron 32	10	9	0	0	0	9	102.8	0.0	0.0	0.0	102.8
Neuron 33	11	8	3	0	0	11	91.4	34.3	0.0	0.0	125.7
Neuron 34	11	5	2	0	0	7	57.1	22.8	0.0	0.0	80.0
Neuron 35	11	3	6	0	0	9	34.3	68.5	0.0	0.0	102.8
Neuron 36	11	12	7	0	0	19	137.1	80.0	0.0	0.0	217.1
Neuron 37	12	9	5	0	0	14	102.8	57.1	0.0	0.0	159.9
Neuron 38	12	10	7	0	0	17	114.2	80.0	0.0	0.0	194.2
Neuron 39	12	7	1	4	0	12	80.0	11.4	45.7	0.0	137.1
Neuron 40	13	5	0	0	0	5	57.1	0.0	0.0	0.0	57.1
Neuron 41	13	8	1	2	0	11	91.4	11.4	22.8	0.0	125.7
Neuron 42	13	9	0	0	0	9	102.8	0.0	0.0	0.0	102.8
Neuron 43	14	8	2	0	0	10	91.4	22.8	0.0	0.0	114.2
Neuron 44	14	8	0	0	0	8	91.4	0.0	0.0	0.0	91.4
Neuron 45	14	10	0	0	0	10	114.2	0.0	0.0	0.0	114.2
Neuron 46	14	16	0	0	0	16	182.8	0.0	0.0	0.0	182.8
Neuron 47	15	9	2	0	0	11	102.8	22.8	0.0	0.0	125.7
Neuron 48	15	9	2	0	0	11	102.8	22.8	0.0	0.0	125.7
Neuron 49	15	18	0	0	0	18	205.6	0.0	0.0	0.0	205.6
Neuron 50	15	5	4	0	0	9	57.1	45.7	0.0	0.0	102.8
MEAN							**85.7**	**25.1**	**2.3**	**0.0**	**113.1**
SD							**44.9**	**28.4**	**8.6**	**0.0**	**42.6**
SE							**6.3**	**4.0**	**1.2**	**0.0**	**6.0**

1ng/ml GDF-5 **Number of intercepts at different levels of neurite field** **Neurite length in µm at different levels of neurite field**

	c slip no.	**Primary**	**Sec**	**Ter**	**Quat**	**Total**	**Primary**	**Sec**	**Ter**	**Quat**	**Total**
Neuron 1	1	4	6	0	0	10	45.7	68.5	0.0	0.0	114.2
Neuron 2	1	16	3	2	0	21	182.8	34.3	22.8	0.0	239.9
Neuron 3	1	11	5	8	0	24	125.7	57.1	91.4	0.0	274.2
Neuron 4	1	23	5	0	0	28	262.8	57.1	0.0	0.0	319.9
Neuron 5	2	6	4	0	0	10	68.5	45.7	0.0	0.0	114.2
Neuron 6	2	6	13	2	0	21	68.5	148.5	22.8	0.0	239.9
Neuron 7	2	25	0	0	0	25	285.6	0.0	0.0	0.0	285.6
Neuron 8	2	3	9	0	0	12	34.3	102.8	0.0	0.0	137.1
Neuron 9	3	7	5	0	0	12	80.0	57.1	0.0	0.0	137.1
Neuron 10	3	7	13	0	0	20	80.0	148.5	0.0	0.0	228.5
Neuron 11	3	6	10	0	0	16	68.5	114.2	0.0	0.0	182.8
Neuron 12	3	11	7	2	3	23	125.7	80.0	22.8	34.3	262.8
Neuron 13	4	13	5	0	0	18	148.5	57.1	0.0	0.0	205.6
Neuron 14	4	14	14	5	0	33	159.9	159.9	57.1	0.0	377.0
Neuron 15	4	8	9	0	0	17	91.4	102.8	0.0	0.0	194.2
Neuron 16	5	26	0	0	0	26	297.0	0.0	0.0	0.0	297.0
Neuron 17	5	17	7	0	0	24	194.2	80.0	0.0	0.0	274.2
Neuron 18	5	8	22	0	0	30	91.4	251.3	0.0	0.0	342.7
Neuron 19	6	20	9	0	0	29	228.5	102.8	0.0	0.0	331.3
Neuron 20	6	6	10	6	0	22	68.5	114.2	68.5	0.0	251.3
Neuron 21	6	4	11	7	0	22	45.7	125.7	80.0	0.0	251.3
Neuron 22	6	12	8	12	0	32	137.1	91.4	137.1	0.0	365.6

Neuron 23	7	3	14	6	0	23	34.3	159.9	68.5	0.0	262.8
Neuron 24	7	7	5	0	0	12	80.0	57.1	0.0	0.0	137.1
Neuron 25	7	2	9	4	0	15	22.8	102.8	45.7	0.0	171.4
Neuron 26	7	10	5	0	0	15	114.2	57.1	0.0	0.0	171.4
Neuron 27	8	9	7	0	0	16	102.8	80.0	0.0	0.0	182.8
Neuron 28	8	5	6	0	0	11	57.1	68.5	0.0	0.0	125.7
Neuron 29	8	9	16	0	0	25	102.8	182.8	0.0	0.0	285.6
Neuron 30	9	10	7	2	0	19	114.2	80.0	22.8	0.0	217.1
Neuron 31	9	12	2	1	0	15	137.1	22.8	11.4	0.0	171.4
Neuron 32	10	6	8	0	0	14	68.5	91.4	0.0	0.0	159.9
Neuron 33	10	10	11	0	0	21	114.2	125.7	0.0	0.0	239.9
Neuron 34	10	15	9	9	0	33	171.4	102.8	102.8	0.0	377.0
Neuron 35	11	4	8	0	0	12	45.7	91.4	0.0	0.0	137.1
Neuron 36	11	13	5	0	0	18	148.5	57.1	0.0	0.0	205.6
Neuron 37	11	4	14	4	0	22	45.7	159.9	45.7	0.0	251.3
Neuron 38	11	8	7	0	0	15	91.4	80.0	0.0	0.0	171.4
Neuron 39	12	13	13	4	0	30	148.5	148.5	45.7	0.0	342.7
Neuron 40	12	5	13	0	0	18	57.1	148.5	0.0	0.0	205.6
Neuron 41	12	7	15	0	0	22	80.0	171.4	0.0	0.0	251.3
Neuron 42	12	7	13	4	0	24	80.0	148.5	45.7	0.0	274.2
Neuron 43	13	15	8	0	0	23	171.4	91.4	0.0	0.0	262.8
Neuron 44	13	10	6	0	0	16	114.2	68.5	0.0	0.0	182.8
Neuron 45	13	13	19	8	0	40	148.5	217.1	91.4	0.0	457.0
Neuron 46	14	12	4	8	0	24	137.1	45.7	91.4	0.0	274.2
Neuron 47	14	24	8	0	0	32	274.2	91.4	0.0	0.0	365.6
Neuron 48	14	5	8	0	0	13	57.1	91.4	0.0	0.0	148.5
Neuron 49	15	4	13	0	0	17	45.7	148.5	0.0	0.0	194.2
Neuron 50	15	9	12	4	1	26	102.8	137.1	45.7	11.4	297.0
MEAN							**115.2**	**100.5**	**22.4**	**0.9**	**239.0**
SD							**67.4**	**51.7**	**35.3**	**5.1**	**79.3**
SE							**9.5**	**7.3**	**5.0**	**0.7**	**11.2**

10ng/ml GDF-5 **Number of intercepts at different levels of neurite field** **Neurite length in µm at different levels of neurite field**

	c slip no.	Primary	Sec	Ter	Quat	Total	Primary	Sec	Ter	Quat	Total
Neuron 1	1	12	6	14	0	32	137.1	68.5	159.9	0.0	365.6
Neuron 2	1	7	4	3	0	14	80.0	45.7	34.3	0.0	159.9
Neuron 3	1	8	2	3	3	16	91.4	22.8	34.3	34.3	182.8
Neuron 4	1	4	12	5	0	21	45.7	137.1	57.1	0.0	239.9
Neuron 5	2	10	8	0	0	18	114.2	91.4	0.0	0.0	205.6
Neuron 6	2	7	8	0	0	15	80.0	91.4	0.0	0.0	171.4
Neuron 7	2	9	3	0	0	12	102.8	34.3	0.0	0.0	137.1
Neuron 8	3	4	7	0	0	11	45.7	80.0	0.0	0.0	125.7
Neuron 9	3	8	6	0	0	14	91.4	68.5	0.0	0.0	159.9
Neuron 10	3	12	0	0	0	12	137.1	0.0	0.0	0.0	137.1
Neuron 11	3	7	15	0	0	22	80.0	171.4	0.0	0.0	251.3
Neuron 12	3	10	10	1	0	21	114.2	114.2	11.4	0.0	239.9
Neuron 13	4	14	5	0	0	19	159.9	57.1	0.0	0.0	217.1
Neuron 14	4	18	13	0	0	31	205.6	148.5	0.0	0.0	354.1
Neuron 15	4	10	0	0	0	10	114.2	0.0	0.0	0.0	114.2
Neuron 16	5	2	14	6	0	22	22.8	159.9	68.5	0.0	251.3
Neuron 17	5	4	4	0	0	8	45.7	45.7	0.0	0.0	91.4
Neuron 18	5	12	11	0	0	23	137.1	125.7	0.0	0.0	262.8
Neuron 19	5	5	8	0	0	13	57.1	91.4	0.0	0.0	148.5
Neuron 20	6	23	0	0	0	23	262.8	0.0	0.0	0.0	262.8
Neuron 21	6	8	5	0	0	13	91.4	57.1	0.0	0.0	148.5
Neuron 22	6	3	18	0	0	21	34.3	205.6	0.0	0.0	239.9
Neuron 23	6	12	2	0	0	14	137.1	22.8	0.0	0.0	159.9
Neuron 24	7	25	7	0	0	32	285.6	80.0	0.0	0.0	365.6
Neuron 25	7	7	11	4	0	22	80.0	125.7	45.7	0.0	251.3
Neuron 26	7	20	0	0	0	20	228.5	0.0	0.0	0.0	228.5
Neuron 27	8	4	24	0	0	28	45.7	274.2	0.0	0.0	319.9
Neuron 28	8	13	4	0	0	17	148.5	45.7	0.0	0.0	194.2
Neuron 29	8	29	0	0	0	29	331.3	0.0	0.0	0.0	331.3
Neuron 30	8	34	12	0	0	46	388.4	137.1	0.0	0.0	525.5
Neuron 31	9	14	4	0	0	18	159.9	45.7	0.0	0.0	205.6
Neuron 32	9	24	4	0	0	28	274.2	45.7	0.0	0.0	319.9
Neuron 33	9	12	0	0	0	12	137.1	0.0	0.0	0.0	137.1
Neuron 34	9	15	8	0	0	23	171.4	91.4	0.0	0.0	262.8
Neuron 35	10	32	0	0	0	32	365.6	0.0	0.0	0.0	365.6
Neuron 36	10	14	10	15	0	39	159.9	114.2	171.4	0.0	445.5
Neuron 37	11	6	13	7	0	26	68.5	148.5	80.0	0.0	297.0
Neuron 38	11	5	11	16	5	37	57.1	125.7	182.8	57.1	422.7
Neuron 39	11	16	14	0	0	30	182.8	159.9	0.0	0.0	342.7
Neuron 40	12	10	6	0	0	16	114.2	68.5	0.0	0.0	182.8
Neuron 41	12	11	34	0	0	45	125.7	388.4	0.0	0.0	514.1
Neuron 42	12	8	6	0	0	14	91.4	68.5	0.0	0.0	159.9
Neuron 43	13	3	9	5	0	17	34.3	102.8	57.1	0.0	194.2
Neuron 44	13	12	20	0	0	32	137.1	228.5	0.0	0.0	365.6
Neuron 45	13	15	4	5	0	24	171.4	45.7	57.1	0.0	274.2
Neuron 46	14	21	16	0	0	37	239.9	182.8	0.0	0.0	422.7
Neuron 47	14	20	14	16	0	50	228.5	159.9	182.8	6.0	571.2
Neuron 48	15	6	10	0	0	16	68.5	114.2	0.0	0.0	182.8
Neuron 49	15	10	7	2	0	19	114.2	80.0	22.8	0.0	217.1
Neuron 50	15	16	4	0	0	20	182.8	45.7	0.0	0.0	228.5
MEAN							**139.6**	**94.4**	**23.3**	**1.9**	**259.1**
SD							**86.3**	**76.7**	**49.6**	**9.3**	**112.4**
SE							**12.2**	**10.8**	**7.0**	**1.3**	**15.9**

12.3.5 MEASUREMENT OF NEURITE BRANCHING

Control **Number of nodes at different levels of neurite field**

	c slip no.	Primary	Sec	Ter	Quat	Total
Neuron 1	1	3	0	0	0	3
Neuron 2	1	1	1	1	0	3
Neuron 3	1	4	1	0	0	5
Neuron 4	1	2	0	0	0	2
Neuron 5	2	4	1	2	0	7
Neuron 6	2	2	0	0	0	2
Neuron 7	2	2	1	0	0	3
Neuron 8	3	3	3	1	0	7
Neuron 9	3	3	1	0	0	4
Neuron 10	3	3	1	0	0	4
Neuron 11	3	4	0	0	0	4
Neuron 12	4	3	1	0	0	4
Neuron 13	4	2	2	0	0	4
Neuron 14	4	5	1	0	0	6
Neuron 15	4	2	1	0	0	3
Neuron 16	5	3	1	0	0	4
Neuron 17	5	2	0	0	0	2
Neuron 18	5	2	2	0	0	4
Neuron 19	5	1	0	0	0	1
Neuron 20	5	2	1	1	0	4
Neuron 21	6	2	0	0	0	2
Neuron 22	6	2	1	0	0	3
Neuron 23	6	3	0	0	0	3
Neuron 24	6	3	0	0	0	3
Neuron 25	6	2	0	0	0	2
Neuron 26	7	2	1	0	0	3
Neuron 27	8	2	0	0	0	2
Neuron 28	8	2	0	0	0	2
Neuron 29	9	2	1	0	0	3
Neuron 30	9	3	0	0	0	3
Neuron 31	9	3	0	0	0	3
Neuron 32	10	2	0	0	0	2
Neuron 33	11	3	1	0	0	4
Neuron 34	11	2	1	0	0	3
Neuron 35	11	2	1	0	0	3
Neuron 36	11	2	1	0	0	3
Neuron 37	12	2	1	0	0	3
Neuron 38	12	1	1	0	0	2
Neuron 39	12	4	1	1	0	6
Neuron 40	13	2	0	0	0	2
Neuron 41	13	2	1	1	0	4
Neuron 42	13	2	0	0	0	2
Neuron 43	14	3	1	0	0	4
Neuron 44	14	2	0	0	0	2
Neuron 45	14	3	0	0	0	3
Neuron 46	14	4	0	0	0	4

Neuron 47	15	2	1	0	0	3
Neuron 48	15	3	1	0	0	4
Neuron 49	15	3	0	0	0	3
Neuron 50	15	2	2	0	0	4
MEAN		**2.5**	**0.7**	**0.1**	**0.0**	**3.3**
SD		**0.8**	**0.7**	**0.4**	**0.0**	**1.3**
SE		**0.1**	**0.1**	**0.1**	**0.0**	**0.2**

1ng/ml GDF-5 **Number of nodes at different levels of neurite field**

	c slip no.	Primary	Sec	Ter	Quat	Total
Neuron 1	1	2	1	1	0	4
Neuron 2	1	5	1	1	0	7
Neuron 3	1	5	1	1	0	7
Neuron 4	1	6	2	0	0	8
Neuron 5	2	5	2	0	0	7
Neuron 6	2	4	3	1	0	8
Neuron 7	2	4	0	0	0	4
Neuron 8	2	3	3	0	0	6
Neuron 9	3	2	1	0	0	3
Neuron 10	3	4	3	0	0	7
Neuron 11	3	2	2	0	0	4
Neuron 12	3	3	2	1	1	7
Neuron 13	4	4	1	0	0	5
Neuron 14	4	5	3	1	0	9
Neuron 15	4	3	1	0	0	4
Neuron 16	5	2	0	0	0	2
Neuron 17	5	4	1	0	0	5
Neuron 18	5	5	3	0	0	8
Neuron 19	6	2	3	0	0	5
Neuron 20	6	3	2	1	0	6
Neuron 21	6	2	2	1	0	5
Neuron 22	6	4	1	1	2	8
Neuron 23	7	3	2	2	1	8
Neuron 24	7	4	1	0	0	5
Neuron 25	7	2	2	1	0	5
Neuron 26	7	3	1	0	0	4
Neuron 27	8	3	1	0	0	4
Neuron 28	8	3	1	0	0	4
Neuron 29	8	2	2	0	0	4
Neuron 30	9	5	2	1	0	8
Neuron 31	9	5	1	1	0	7
Neuron 32	10	4	2	0	0	6
Neuron 33	10	2	2	0	0	4
Neuron 34	10	2	2	1	0	5
Neuron 35	11	6	1	0	0	7
Neuron 36	11	2	1	0	0	3
Neuron 37	11	4	2	2	0	8
Neuron 38	11	6	1	0	0	7
Neuron 39	12	2	2	1	0	5
Neuron 40	12	5	2	0	0	7
Neuron 41	12	2	2	0	0	4
Neuron 42	12	2	2	1	1	6
Neuron 43	13	3	2	0	0	5
Neuron 44	13	3	1	0	0	4
Neuron 45	13	2	3	1	0	6
Neuron 46	14	4	1	1	0	6
Neuron 47	14	4	1	0	0	5
Neuron 48	14	3	2	0	0	5
Neuron 49	15	3	2	0	0	5
Neuron 50	15	3	2	2	1	8
MEAN		**3.4**	**1.7**	**0.5**	**0.1**	**5.7**
SD		**1.2**	**0.8**	**0.6**	**0.4**	**1.7**
SE		**0.2**	**0.1**	**0.1**	**0.1**	**0.2**

10ng/ml GDF-5 **Number of nodes at different levels of neurite field**

	c slip no.	Primary	Sec	Ter	Quat	Total
Neuron 1	1	3	2	2	0	7
Neuron 2	1	4	1	1	0	6
Neuron 3	1	4	1	1	1	7
Neuron 4	1	3	3	2	0	8
Neuron 5	2	4	2	0	0	6
Neuron 6	2	2	1	0	0	3
Neuron 7	2	2	1	0	0	3
Neuron 8	3	2	4	0	0	6
Neuron 9	3	4	1	1	0	6
Neuron 10	3	3	0	0	0	3
Neuron 11	3	2	2	0	0	4
Neuron 12	3	4	2	1	0	7
Neuron 13	4	3	1	0	0	4
Neuron 14	4	5	2	0	0	7
Neuron 15	4	2	0	0	0	2
Neuron 16	5	3	2	2	0	7
Neuron 17	5	4	1	0	0	5
Neuron 18	5	4	2	0	0	6
Neuron 19	5	3	2	0	0	5
Neuron 20	6	2	0	0	0	2
Neuron 21	6	2	1	0	0	3
Neuron 22	6	3	2	0	0	5
Neuron 23	6	2	1	0	0	3
Neuron 24	7	2	2	0	0	4
Neuron 25	7	4	2	1	0	7
Neuron 26	7	2	0	0	0	2
Neuron 27	8	2	2	0	0	4
Neuron 28	8	3	1	0	0	4
Neuron 29	8	2	0	0	0	2
Neuron 30	8	3	1	0	0	4
Neuron 31	9	4	1	0	0	5
Neuron 32	9	3	1	0	0	4
Neuron 33	9	1	0	0	0	1
Neuron 34	9	3	1	0	0	4
Neuron 35	10	2	0	0	0	2
Neuron 36	10	4	2	2	0	8
Neuron 37	11	2	2	1	0	5
Neuron 38	11	2	2	1	1	6
Neuron 39	11	4	1	0	0	5
Neuron 40	12	3	1	0	0	4
Neuron 41	12	4	2	0	0	6
Neuron 42	12	4	2	0	0	6
Neuron 43	13	4	2	1	0	7
Neuron 44	13	2	2	0	0	4
Neuron 45	13	4	1	1	0	6
Neuron 46	13	5	2	0	0	7
Neuron 47	14	6	3	2	0	11
Neuron 48	14	3	2	0	0	5
Neuron 49	15	3	2	1	0	6
Neuron 50	15	2	2	0	0	4
MEAN		**3.1**	**1.5**	**0.4**	**0.0**	**5.0**
SD		**1.0**	**0.9**	**0.7**	**0.2**	**2.0**
SE		**0.1**	**0.1**	**0.1**	**0.0**	**0.3**

12.3.6 **MEASUREMENT OF SOMAL AREA**

	Control			1ng/ml GDF-5		10ng/ml GDF-5	
	c slip no.	Points	Somal area (μm^2)	Points	Somal area (μm^2)	Points	Somal area (μm^2)
Neuron 1	1	7	242.9	7	242.9	8	277.6
Neuron 2	1	6	208.2	7	242.9	9	312.3
Neuron 3	1	6	208.2	9	312.3	8	277.6
Neuron 4	1	7	242.9	13	451.1	10	347
Neuron 5	2	7	242.9	8	277.6	9	312.3
Neuron 6	2	7	242.9	9	312.3	8	277.6
Neuron 7	2	8	277.6	10	347	11	381.7
Neuron 8	3	4	138.8	9	312.3	8	277.6
Neuron 9	3	7	242.9	10	347	9	312.3
Neuron 10	3	7	242.9	8	277.6	10	347

Neuron 11	3	7	242.9	10	347	10	347
Neuron 12	4	7	242.9	12	416.4	10	347
Neuron 13	4	8	277.6	10	347	8	277.6
Neuron 14	4	8	277.6	13	451.1	12	416.4
Neuron 15	4	7	242.9	9	312.3	8	277.6
Neuron 16	5	6	208.2	10	347	11	381.7
Neuron 17	5	7	242.9	11	381.7	12	416.4
Neuron 18	5	8	277.6	8	277.6	11	381.7
Neuron 19	5	7	242.9	11	381.7	9	312.3
Neuron 20	5	8	277.6	10	347	13	451.1
Neuron 21	6	7	242.9	11	381.7	10	347
Neuron 22	6	6	208.2	14	485.8	10	347
Neuron 23	6	7	242.9	10	347	8	277.6
Neuron 24	6	7	242.9	9	312.3	14	485.8
Neuron 25	6	8	277.6	11	381.7	10	347
Neuron 26	7	7	242.9	8	277.6	8	277.6
Neuron 27	8	5	173.5	11	381.7	14	485.8
Neuron 28	8	5	173.5	7	242.9	11	381.7
Neuron 29	9	7	242.9	13	451.1	15	520.5
Neuron 30	9	4	138.8	10	347	9	312.3
Neuron 31	9	6	208.2	9	312.3	9	312.3
Neuron 32	10	7	242.9	8	277.6	10	347
Neuron 33	11	8	277.6	10	347	9	312.3
Neuron 34	11	8	277.6	12	416.4	9	312.3
Neuron 35	11	6	208.2	12	416.4	11	381.7
Neuron 36	11	7	242.9	11	381.7	12	416.4
Neuron 37	12	9	312.3	12	416.4	10	347
Neuron 38	12	9	312.3	12	416.4	10	347
Neuron 39	12	7	242.9	10	347	11	381.7
Neuron 40	13	8	277.6	9	312.3	12	416.4
Neuron 41	13	6	208.2	10	347	13	451.1
Neuron 42	13	10	347	9	312.3	9	312.3
Neuron 43	14	8	277.6	10	347	13	451.1
Neuron 44	14	9	312.3	9	312.3	10	347
Neuron 45	14	8	277.6	16	555.2	8	277.6
Neuron 46	14	10	347	14	485.8	7	242.9
Neuron 47	15	7	242.9	10	347	12	416.4
Neuron 48	15	6	208.2	8	277.6	9	312.3
Neuron 49	15	8	277.6	8	277.6	10	347
Neuron 50	15	7	242.9	10	347	11	381.7
MEAN			**247.1**		**351.9**		**352.6**
SD			**43.0**		**67.2**		**63.6**
SE			**6.1**		**9.5**		**9.0**

12.4 APPENDIX D (CHAPTER 7)

12.4.1 COUNTS OF TH-, TUJ-1- and DAPI-POSITIVE CELLS AFTER GDF-5 TREATMENT FOR 3 DIV IN E14 VM CULTURES.

	TH-positive cells/field		Average no./field		TUJ-1-positive cells/field		Average no./field		DAPI-positive cells/field		Average no./field	
	Control	10ng.ml GDF-5			Control	10ng.ml GDF-5			Control	10ng.ml GDF-5		
1.1	1	1			24	8			30	10		
1.2	2	4			13	23			24	33		
1.3	0	2			25	13			30	17		
1.4	1	2			19	35			22	37		
1.5	0	3	0.8	2.4	15	12	19.2	18.2	20	17	25.2	22.8
2.1	0	1			20	14			29	17		
2.2	2	3			18	15			29	19		
2.3	1	2			16	20			29	30		
2.4	2	2			15	29			28	38		
2.5	0	1	1	1.8	20	10	17.8	17.6	37	14	30.4	23.6
3.1	1	1			17	16			25	21		
3.2	6	6			30	20			33	28		
3.3	0	1			20	18			26	25		
3.4	0	2			18	6			25	10		
3.5	2	1	1.8	2.2	16	10	20.2	14	26	15	27	19.8
4.1	0	3			10	14			12	16		
4.2	1	1			12	7			24	11		
4.3	1	1			14	10			24	11		
4.4	0	3			5	24			25	37		
4.5	1	2	0.6	2	17	20	11.6	15	26	22	22.2	19.4
5.1	2	1			8	9			17	14		
5.2	0	4			22	9			28	13		
5.3	1	1			9	20			14	24		
5.4	1	4			20	12			22	23		
5.5	0	2	0.8	2.4	15	12	14.8	12.4	20	26	20.2	20
6.1	2	2			22	21			26	26		
6.2	0	4			13	25			16	36		
6.3	1	5			7	23			11	29		
6.4	0	1			12	13			15	19		
6.5	3	0	1.2	2.4	27	11	16.2	18.6	33	15	20.2	25
7.1	2	1			43	33			48	36		
7.2	2	3			46	56			49	59		
7.3	3	3			64	64			68	68		
7.4	2	5			47	41			49	44		
7.5	2	4	2.2	3.2	41	58	48.2	50.4	45	67	51.8	54.8
8.1	1	5			33	54			39	58		
8.2	1	3			37	41			41	45		
8.3	2	4			68	41			76	44		
8.4	1	5			45	59			52	69		
8.5	1	2	1.2	3.8	40	29	44.6	44.8	43	31	50.2	49.4
9.1	1	4			41	43			45	47		
9.2	1	3			57	81			65	88		
9.3	1	2			41	44			48	49		
9.4	1	5			68	57			75	61		
9.5	1	3	1	3.4	31	36	47.6	52.2	34	39	53.4	56.8
MEAN			**1.2**	**2.6**			**26.7**	**27.0**			**33.4**	**32.4**
S.D.			**0.5**	**0.7**			**15.3**	**16.8**			**14.2**	**16.2**
S.E.			**0.3**	**0.4**			**8.8**	**9.7**			**8.2**	**9.3**

12.4.2 PERCENTAGE OF TUJ-1- and DAPI-POSITIVE CELLS THAT EXPRESS TH AFTER GDF-5 TREATMENT FOR 3 DIV IN E14 VM CULTURES.

	%TH OF TUJ-1-positive cells				%TH-DAPI-positive cells			
	Control	10ng/ml GDF-5			Control	10ng/ml GDF-5		
1.1	4.2	12.5			3.3	10.0		
1.2	15.4	17.4			8.3	12.1		
1.3	0.0	15.4			0.0	11.8		
1.4	5.3	5.7			4.5	5.4		
1.5	0.0	25.0	5.0	15.2	0.0	17.6	3.2	11.4
2.1	0.0	7.1			0.0	5.9		
2.2	11.1	20.0			6.9	15.8		
2.3	6.3	10.0			3.4	6.7		
2.4	13.3	6.9			7.1	5.3		
2.5	0.0	10.0	6.1	10.8	0.0	7.1	3.5	8.1
3.1	5.9	6.3			4.0	4.8		
3.2	20.0	30.0			18.2	21.4		
3.3	0.0	5.6			0.0	4.0		
3.4	0.0	33.3			0.0	20.0		
3.5	12.5	10.0	7.7	17.0	7.7	6.7	6.0	11.4
4.1	0.0	21.4			0.0	18.8		
4.2	8.3	14.3			4.2	9.1		
4.3	7.1	10.0			4.2	9.1		
4.4	0.0	12.5			0.0	8.1		

4.5	5.9	10.0	4.3	13.6	3.8	9.1	2.4	10.8
5.1	25.0	11.1			11.8	7.1		
5.2	0.0	44.4			0.0	30.8		
5.3	11.1	5.0			7.1	4.2		
5.4	5.0	33.3			4.5	17.4		
5.5	0.0	16.7	8.2	22.1	0.0	7.7	4.7	13.4
6.1	9.1	9.5			7.7	7.7		
6.2	0.0	16.0			0.0	11.1		
6.3	14.3	21.7			9.1	17.2		
6.4	0.0	7.7			0.0	5.3		
6.5	11.1	0.0	6.9	11.0	9.1	0.0	5.2	8.3
7.1	4.7	3.0			4.2	2.8		
7.2	4.3	5.4			4.1	5.1		
7.3	4.7	4.7			4.4	4.4		
7.4	4.3	12.2			4.1	11.4		
7.5	4.9	6.9	4.6	6.4	4.4	6.0	4.2	5.9
8.1	3.0	9.3			2.6	8.6		
8.2	2.7	7.3			2.4	6.7		
8.3	2.9	9.8			2.6	9.1		
8.4	2.2	8.5			1.9	7.2		
8.5	2.5	6.9	2.7	8.3	2.3	6.5	2.4	7.6
9.1	2.4	9.3			2.2	8.5		
9.2	1.8	3.7			1.5	3.4		
9.3	2.4	4.5			2.1	4.1		
9.4	1.5	8.8			1.3	8.2		
9.5	3.2	8.3	2.3	6.9	2.9	7.7	2.0	6.4
MEAN			**5.3**	**12.4**			**3.7**	**9.3**
SD			**2.1**	**5.1**			**1.4**	**2.6**
SE			**1.2**	**3.0**			**0.8**	**1.5**

12.4.3 PERCENTAGE OF TUJ-1-POSITIVE CELLS THAT EXPRESS TH AFTER GDF-5, WNT-5A OR GDF-5 AND WNT-5A TREATMENT FOR 3 DIV IN E14 VM CULTURES.

%TH OF TUJ-1-positive cells

C.SLIP NO./ FIELD NO.	**Control**	**10ng/ml GDF-5**	**1:100 Wnt-5a**	**1:100 Wnt-5a control**	**GDF-5 (10ng/ml) + Wnt-5a (1:100)**	**GDF-5 (10ng/ml) + Wnt-5a control**	**Average percentage**					
1.1	4.2	12.5	8.0	11.1	16.7	0.0						
1.2	15.4	17.4	14.3	0.0	5.0	13.3						
1.3	0.0	15.4	3.6	0.0	28.6	40.0						
1.4	5.3	5.7	7.7	5.6	12.5	21.4						
1.5	0.0	25.0	28.6	7.7	10.0	16.7	5.0	15.2	12.4	4.9	14.5	18.3
2.1	0.0	7.1	11.1	20.0	33.3	6.7						
2.2	11.1	20.0	13.6	0.0	33.3	8.3						
2.3	6.3	10.0	9.7	9.5	11.8	6.7						
2.4	13.3	6.9	10.0	0.0	13.3	13.3						
2.5	0.0	10.0	9.5	5.0	5.9	0.0	6.1	10.8	10.8	6.9	19.5	7.0
3.1	5.9	6.3	22.2	0.0	30.0	11.4						
3.2	20.0	30.0	9.1	0.0	10.0	7.1						
3.3	0.0	5.6	7.1	0.0	14.3	18.2						
3.4	0.0	33.3	5.9	4.8	8.3	5.6						
3.5	12.5	10.0	10.0	13.8	5.6	0.0	7.7	17.0	10.9	3.7	13.6	8.5
4.1	0.0	21.4	7.7	9.1	23.5	4.3						
4.2	8.3	14.3	16.7	0.0	12.5	22.2						
4.3	7.1	10.0	9.5	8.3	12.5	6.7						
4.4	0.0	12.5	19.0	20.0	15.4	22.2						
4.5	5.9	10.0	0.0	0.0	11.1	6.7	4.3	13.6	10.6	7.5	15.0	12.4
5.1	25.0	11.1	16.7	8.3	20.0	16.7						
5.2	0.0	44.4	5.6	33.3	26.9	5.6						
5.3	11.1	5.0	6.7	6.7	44.4	33.3						
5.4	5.0	33.3	25.0	0.0	0.0	0.0						
5.5	0.0	16.7	16.7	0.0	3.7	4.3	8.2	22.1	14.1	9.7	19.0	12.0
6.1	9.1	9.5	8.3	12.5	20.0	8.7						
6.2	0.0	16.0	9.1	10.5	0.0	5.9						
6.3	14.3	21.7	20.0	22.2	7.7	0.0						
6.4	0.0	7.7	4.2	0.0	5.3	4.5						
6.5	11.1	0.0	17.6	0.0	0.0	0.0	6.9	11.0	11.8	9.0	6.6	3.8
7.1	4.7	3.0										
7.2	4.3	5.4										
7.3	4.7	4.7										
7.4	4.3	12.2										
7.5	4.9	6.9					4.6	6.4				
8.1	3.0	9.3										
8.2	2.7	7.3										
8.3	2.9	9.8										
8.4	2.2	8.5										
8.5	2.5	6.9					2.7	8.3				
9.1	2.4	9.3										
9.2	1.8	3.7										
9.3	2.4	4.5										
9.4	1.5	8.8										
9.5	3.2	8.3					2.3	6.9				
MEAN							**5.3**	**12.4**	**12.6**	**6.0**	**12.6**	**8.9**
SD							**2.1**	**5.1**	**2.5**	**3.4**	**7.0**	**6.0**
SE							**1.2**	**3.0**	**1.8**	**2.4**	**5.0**	**4.3**

12.4.4 PERCENTAGE OF DAPI-POSITIVE CELLS THAT EXPRESS TH AFTER GDF-5, WNT-5A OR GDF-5 AND WNT-5A TREATMENT FOR 3 DIV IN E14 VM CULTURES.

%TH OF TUJ-1-positive cells

C.SLIP NO./ FIELD NO.	Control	10ng/ml GDF-5	1:100 Wnt-5a	1:100 Wnt-5a control	GDF-5 (10ng/ml) + Wnt-5a (1:100)	GDF-5 (10ng/ml) + Wnt-5a control	Average percentage					
1.1	3.3	10.0	6.7	6.7	13.0	0.0						
1.2	8.3	12.1	10.0	0.0	4.2	10.5						
1.3	0.0	11.8	3.1	0.0	18.2	32.0						
1.4	4.5	5.4	5.6	4.2	5.3	18.8						
1.5	0.0	17.6	22.2	4.3	8.7	13.3	3.2	11.4	9.5	3.0	9.9	14.9
2.1	0.0	5.9	8.3	14.3	22.2	5.9						
2.2	6.9	15.8	10.3	0.0	9.1	6.3						
2.3	3.4	6.7	8.8	7.4	11.1	4.8						
2.4	7.1	5.3	7.1	0.0	11.8	11.1						
2.5	0.0	7.1	6.5	3.8	5.0	0.0	3.5	8.1	8.2	5.1	11.8	5.6
3.1	4.0	4.8	20.0	0.0	17.6	12.1						
3.2	18.2	21.4	8.3	0.0	6.7	6.3						
3.3	0.0	4.0	6.3	0.0	13.3	18.2						
3.4	0.0	20.0	4.5	4.3	5.9	4.0						
3.5	7.7	6.7	6.7	11.8	4.5	0.0	6.0	11.4	9.2	3.2	9.6	8.1
4.1	0.0	18.8	7.1	6.3	18.2	3.3						
4.2	4.2	9.1	12.5	0.0	7.1	18.2						
4.3	4.2	9.1	7.4	5.6	5.0	4.8						
4.4	0.0	8.1	13.3	11.8	14.3	14.8						
4.5	3.8	9.1	0.0	0.0	10.0	5.0	2.4	10.8	8.1	4.7	10.9	9.2
5.1	11.8	7.1	14.8	5.6	15.0	14.3						
5.2	0.0	30.8	3.6	12.5	19.4	4.0						
5.3	7.1	4.2	5.9	5.6	34.8	22.0						
5.4	4.5	17.4	20.0	0.0	0.0	0.0						
5.5	0.0	7.7	8.3	0.0	3.1	4.2	4.7	13.4	10.5	4.7	14.5	8.9
6.1	7.7	7.7	6.7	7.1	13.3	6.5						
6.2	0.0	11.1	5.9	8.3	0.0	4.8						
6.3	9.1	17.2	9.1	16.0	6.7	0.0						
6.4	0.0	5.3	2.8	0.0	3.7	4.2						
6.5	9.1	0	15	0	0	0	5.2	8.3	7.9	6.3	4.7	3.1
7.1	4.2	2.8										
7.2	4.1	5.1										
7.3	4.4	4.4										
7.4	4.1	11.4							15.0	0.0	0.0	0.0
7.5	4.4	6.0					4.2	5.9				
8.1	2.6	8.6										
8.2	2.4	6.7										
8.3	2.6	9.1										
8.4	1.9	7.2										
8.5	2.3	6.5					2.4	7.6				
9.1	2.2	8.5										
9.2	1.5	3.4										
9.3	2.1	4.1										
9.4	1.3	8.2										
9.5	2.9	7.7					2.0	6.4				
MEAN							**3.7**	**9.3**	**9.8**	**3.9**	**8.8**	**7.1**
SD							**1.4**	**2.6**	**2.5**	**2.0**	**4.9**	**4.8**
SE							**0.8**	**1.5**	**1.8**	**1.4**	**3.4**	**3.4**

12.5 APPENDIX E (CHAPTER 8)

12.5.1 AVERAGE VALUES OF NEUROSPHERE DIAMETER AT 2, 4, 6 DIV (MAGNIFICATION = x 232.5)

FLASK NO./ FIELD NO.	2 DIV E12	2 DIV E13	2 DIV E14	4 DIV E12	4 DIV E13	4 DIV E14	6 DIV E12	6 DIV E13	6 DIV E14
1.1	1.75	1.20	1.15	2.88	4.55	1.62	3.18	3.07	1.65
1.2	1.33	1.43	0.87	2.68	4.87	2.00	3.04	3.28	1.68
1.3	1.55	1.44	0.95	3.30	4.13	1.95	2.94	2.73	1.70
1.4	1.80	1.90	1.15	2.80	4.38	1.77	2.98	2.52	2.17
1.5	1.53	1.26	1.07	4.23	3.67	1.48	2.80	2.92	1.97
1.6	1.04	1.80	1.02	2.78	3.72	1.62	3.48	2.63	2.10
2.1	2.05	1.15	0.83	3.70	2.47	1.38	3.10	2.45	2.10
2.2	1.42	1.08	0.83	3.87	2.75	1.55	2.80	2.33	2.07
2,3	1.57	1.25	1.05	4.67	3.23	1.58	3.16	2.43	2.07
2.4	1.87	2.00	0.93	4.25	2.28	1.65	2.46	2.03	1.92
2.5	1.75	1.28	0.93	4.00	2.50	1.55	2.94	2.40	1.62
2.6	2.02	1.02	1.50	4.78	2.87	1.53	2.40	2.47	1.73
3.1	2.95	1.63	0.73	3.53	5.52		3.20	2.32	1.27
3.2	1.43	1.37	0.83	2.63	4.33		3.42	3.45	1.52
3.3	1.59	1.28	0.92	3.81	3.65		2.62	3.13	1.47
3.4	1.76	1.55	0.88	3.72	4.90		2.90	3.67	1.53
3.5	1.46	1.65	0.85	3.98	3.88		2.73	2.77	1.68
3.6	1.45	1.48	1.05	4.65	4.45		2.89	3.47	1.55
MEAN D (cm)	1.68	1.43	0.98	3.68	3.79	1.64	2.95	2.78	1.77
MEAN D (µm)	16831.75	14324.07	9750.00	36806.88	37861.11	16402.78	29461.90	27814.81	17657.41
ACTUAL D (µm)	**72.39**	**61.61**	**41.94**	**158.31**	**162.84**	**70.55**	**316.79**	**299.08**	**189.86**
SD (cm)	0.40	0.28	0.17	0.71	0.94	0.18	0.29	0.47	0.26
SD (µm)	4025.23	2786.89	1749.18	7085.10	9433.68	1819.45	2924.00	4679.22	2639.24
ACTUAL SD (µm)	**17.31**	**11.99**	**7.52**	**30.47**	**40.57**	**7.83**	**31.44**	**50.31**	**28.38**
ACTUAL SEM (µm)	**10.00**	**6.92**	**4.34**	**17.59**	**23.43**	**5.53**	**18.15**	**29.05**	**16.38**

ACTUAL D = (SD(cm) x 10,000) / MAGNIFICATION

ACTUAL SD = (MEAN D(cm) x 10,000) / MAGNIFICATION

ACTUAL SEM = ACTUAL SD / √3

12.5.2 AVERAGE VALUES OF NEUROSPHERE VOLUME AT 2, 4, 6 DIV (MAGNIFICATION = x 232.5)

FLASK NO./ FIELD NO.	2 DIV E12	2 DIV E13	2 DIV E14	4 DIV E12	4 DIV E13	4 DIV E14	6 DIV E12	6 DIV E13	6 DIV E14
1.1	2.40	1.13	1.04	6.49	16.25	2.05	7.94	7.38	2.14
1.2	1.40	1.61	0.59	5.62	18.59	3.14	7.25	8.46	2.22
1.3	1.89	1.63	0.71	8.55	13.41	2.98	6.79	5.86	2.27
1.4	2.54	2.83	1.04	6.15	15.08	2.45	6.97	4.97	3.69
1.5	1.83	1.25	0.89	14.01	10.55	1.73	6.15	6.68	3.04
1.6	0.85	2.54	0.81	6.07	10.84	2.05	9.52	5.44	3.46
2.1	3.30	1.04	0.55	10.75	4.78	1.50	7.54	4.71	3.46
2.2	1.58	0.92	0.55	11.74	5.94	1.89	6.15	4.27	3.35
2.3	1.93	1.23	0.87	17.13	8.21	1.97	7.84	4.65	3.35
2.4	2.74	3.14	0.68	14.18	4.09	2.14	4.74	3.25	2.88
2.5	2.40	1.29	0.68	12.56	4.91	1.89	6.79	4.52	2.05
2.6	3.19	0.81	1.77	17.96	6.45	1.85	4.52	4.78	2.36
3.1	6.83	2.09	0.42	9.80	23.89	0.00	8.04	4.21	1.26
3.2	1.60	1.47	0.55	5.41	14.74	0.00	9.18	9.34	1.81
3.3	1.97	1.29	0.66	11.42	10.46	0.00	5.37	7.71	1.69
3.4	2.42	1.89	0.61	10.86	18.85	0.00	6.60	10.55	1.85
3.5	1.67	2.14	0.57	12.46	11.84	0.00	5.84	6.01	2.22
3.6	1.65	1.73	0.87	16.97	15.54	0.00	6.54	9.43	1.89
MEAN V (cm^3)	2.34	1.67	0.77	11.01	11.91	1.42	6.88	6.24	2.50
MEAN V (μm^3)	23440.89	16682.38	7689.24	110069.25	119124.84	14239.15	68772.17	62355.89	24991.47
ACTUAL V (μm^3)	**100.82**	**71.75**	**33.07**	**473.42**	**512.36**	**61.24**	**739.49**	**670.49**	**268.73**
SD (cm^3)	1.28	0.66	0.30	4.07	5.60	1.11	1.35	2.12	0.74
SD (μm^3)	12827.31	6607.38	3042.26	40736.66	56048.74	11086.78	13506.21	21195.57	7351.22
ACTUAL SD (μm^3)	**55.17**	**28.42**	**13.08**	**175.21**	**241.07**	**47.69**	**58.09**	**91.16**	**31.62**
ACTUAL SEM(μm^3)	**31.85**	**16.41**	**7.55**	**101.16**	**139.18**	**27.53**	**33.54**	**52.63**	**18.25**

ACTUAL V = (MEAN V(cm^3) x 10,000) / MAGNIFICATION

ACTUAL SD = (MEAN D(cm^3) x 10,000) / MAGNIFICATION

ACTUAL SEM = ACTUAL SD / $\sqrt{3}$

12.5.3 CULTURE CHARACTERISATION OF DIFFERENTIATING NEUROSPHERES AFTER 6 DIV (TUJ-1-1)

	E12				E13				E14			
C.SLIP NO./ FIELD NO.	**TUJ-1(+) cells/ field**	**DAPI/ field**	**%TUJ-1/ DAPI**	**MEAN %**	**TUJ-1(+) cells/ field**	**DAPI/ field**	**%TUJ-1/ DAPI**	**MEAN %**	**TUJ-1(+) cells/ field**	**DAPI/ field**	**%TUJ-1/ DAPI**	**MEAN %**
1.1	11	83	13.3		15	99	15.2		1	79	1.3	
1.2	10	57	17.5		18	100	18.0		1	76	1.3	
1.3	22	103	21.4		10	114	8.8		0	69	0.0	
1.4	14	53	26.4	19.6	15	106	14.2	14.0	2	52	3.8	1.6
2.1	22	98	22.4		12	73	16.4		3	110	2.7	
2.2	21	109	19.3		12	83	14.5		2	107	1.9	
2.3	13	70	18.6		18	120	15.0		5	105	4.8	
2.4	13	63	20.6	20.2	14	75	18.7	16.1	2	77	2.6	3.0
3.1	20	65	30.8		15	106	14.2		1	63	1.6	
3.2	24	84	28.6		10	62	16.1		3	94	3.2	
3.3	21	106	19.8		7	77	9.1		0	88	0.0	
3.4	19	128	14.8	23.5	8	83	9.6	12.3	4	104	3.8	2.2
4.1	12	97	12.4		12	90	13.3		2	97	2.1	
4.2	22	110	20.0		13	84	15.5		0	74	0.0	
4.3	21	115	18.3		14	86	16.3		2	110	1.8	
4.4	16	113	14.2	16.2	17	130	13.1	14.5	3	88	3.4	1.8
5.1	13	120	10.8		12	119	10.1		3	65	4.6	
5.2	21	84	25.0		6	53	11.3		7	51	13.7	
5.3	5	44	11.4		13	119	10.9		3	74	4.1	
5.4	23	101	22.8	17.5	5	68	7.4	9.9	7	50	14.0	9.1
6.1	13	103	12.6		11	82	13.4		9	84	10.7	
6.2	25	130	19.2		12	86	14.0		2	45	4.4	
6.3	25	127	19.7		11	80	13.8		10	111	9.0	
6.4	23	107	21.5	18.3	10	94	10.6	12.9	7	82	8.5	8.2
7.1	20	97	20.6		5	83	6.0		7	81	8.6	
7.2	24	103	23.3		14	87	16.1		4	69	5.8	
7.3	16	96	16.7		11	82	13.4		6	40	15.0	
7.4	17	121	14.0	18.7	12	110	10.9	11.6	4	95	4.2	8.4
8.1	21	97	21.6		8	72	11.1		4	54	7.4	
8.2	23	108	21.3		5	76	6.6		6	84	7.1	
8.3	16	114	14.0		12	121	9.9		5	81	6.2	
8.4	18	92	19.6	19.1	13	80	16.3	11.0	5	50	10.0	7.7
9.1	30	157	19.1		12	63	19.0		8	51	15.7	
9.2	17	97	17.5		15	70	21.4		20	80	25.0	
9.3	27	103	26.2		8	85	9.4		8	66	12.1	
9.4	30	120	25.0	22.0	12	66	18.2	17.0	3	41	7.3	15.0
10.1	20	113	17.7		4	47	8.5		3	49	6.1	
10.2	19	133	14.3		8	56	14.3		5	70	7.1	
10.3	21	140	15.0		6	50	12.0		6	57	10.5	
10.4	24	116	20.7	16.9	10	67	14.9	12.4	4	53	7.5	7.8
11.1	19	80	23.8		9	62	14.5		5	62	8.1	
11.2	19	112	17.0		7	41	17.1		5	54	9.3	
11.3	27	126.0	21.4		8	60	13.3		5	46	10.9	
11.4	20	98.0	20.4	20.6	9	70	12.9	14.4	7	91	7.7	9.0
12.1	18	85.0	21.2		10	72	13.9		7	78	9.0	
12.2	23	109.0	21.1		8	46	17.4		3	38	7.9	
12.3	20	103.0	19.4		10	75	13.3		4	62	6.5	
12.4	13	84.0	15.5	19.3	12	48	25.0	17.4	6	47	12.8	9.0
MEAN				**19.3**				**13.6**				**6.9**
SD				**2.1**				**2.4**				**4.0**
SE				**0.6**				**0.7**				1.2

12.5.4 CULTURE CHARACTERISATION OF DIFFERENTIATING NEUROSPHERES AFTER 6 DIV (MAP-2ab)

	E12				E13				E14			
C.SLIP NO./ FIELD NO.	**MAP2(+) cells/ field**	**DAPI/ field**	**%MAP2/ DAPI**	**MEAN %**	**MAP2(+) cells/ field**	**DAPI/ field**	**%MAP2/ DAPI**	**MEAN %**	**MAP2(+) cells/ field**	**DAPI/ field**	**%MAP2/ DAPI**	**MEAN %**
1.1	10	67	14.9		5	110	4.5		1	110	0.9	
1.2	26	133	19.5		18	89	20.2		3	103	2.9	
1.3	17	110	15.5		12	93	12.9		2	57	3.5	
1.4	7	57	12.3	15.6	8	70	11.4	12.3	4	57	7.0	3.6
2.1	21	140	15.0		13	89	14.6		1	114	0.9	
2.2	16	105	15.2		16	108	14.8		2	104	1.9	
2.3	18	94	19.1		18	83	21.7		3	99	3.0	
2.4	20	140	14.3	15.9	15	80	18.8	17.5	2	83	2.4	2.1
3.1	10	97	10.3		9	127	7.1		1	121	0.8	
3.2	24	142	16.9		14	118	11.9		2	84	2.4	
3.3	22	150	14.7		12	100	12.0		2	106	1.9	
3.4	12	61	19.7	15.4	15	105	14.3	11.3	4	83	4.8	2.5
4.1	17	96	17.7		18	131	13.7		2	68	2.9	
4.2	16	72	22.2		14	114	12.3		3	72	4.2	
4.3	22	108	20.4		9	110	8.2		3	96	3.1	
4.4	19	141	13.5	18.4	15	96	15.6	12.5	2	108	1.9	3.0
5.1	20	103	19.4		5	64	7.8		2	56	3.6	
5.2	9	76	11.8		8	120	6.7		5	109	4.6	
5.3	20	102	19.6		10	84	11.9		3	28	10.7	
5.4	16	104	15.4	16.6	9	66	13.6	10.0	1	68	1.5	5.1
6.1	25	108	23.1		12	81	14.8		3	63	4.8	
6.2	20	83	24.1		10	87	11.5		1	44	2.3	
6.3	18	132	13.6		11	93	11.8		2	32	6.3	
6.4	30	148	20.3	20.3	10	105	9.5	11.9	5	112	4.5	4.4
7.1	28	160	17.5		13	71	18.3		3	56	5.4	
7.2	14	116	12.1		9	69	13.0		3	71	4.2	
7.3	31	180	17.2		7	90	7.8		4	55	7.3	
7.4	13	101	12.9	14.9	8	110	7.3	11.6	1	54	1.9	4.7
8.1	28	132	21.2		18	116	15.5		1	21	4.8	
8.2	25	192	13.0		14	105	13.3		1	67	1.5	
8.3	15	97	15.5		16	111	14.4		1	64	1.6	
8.4	10	107	9.3	14.8	11	80	13.8	14.3	3	82	3.7	2.9
9.1	9	38	23.7		13	111	11.7					
9.2	35	197	17.8		7	101	6.9					
9.3	33	147	22.4		11	93	11.8					
9.4	25	150	16.7	20.1	6	59	10.2	10.2				
10.1	21	110	19.1		10	96	10.4					
10.2	20	103	19.4		9	107	8.4					
10.3	28	143	19.6		5	90	5.6					
10.4	27	167	16.2	18.6	11	80	13.8	9.5				
11.1	22	132	16.7		9	87	10.3					
11.2	21	84	25.0		10	105	9.5					
11.3	21	130	16.2		13	88	14.8					
11.4	27	143	18.9	19.2	8	101	7.9	10.6				
12.1	21	120	17.5		13	81	16.0					
12.2	24	140	17.1		12	105	11.4					
12.3	29	180	16.1		10	81	12.3					
12.4	21	131	16.0	16.7	4	62	6.5	11.6				
MEAN				**17.2**				**11.9**				**3.5**
SD				**2.0**				**2.2**				**1.1**
SE				**0.6**				**0.6**				**0.4**

12.5.5 CULTURE CHARACTERISATION OF DIFFERENTIATING NEUROSPHERES AFTER 6 DIV (GFAP)

	E12				E13				E14			
C.SLIP NO./ FIELD NO.	**GFAP(+) cells/ field**	**DAPI/ field**	**%GFAP/ DAPI**	**MEAN %**	**GFAP(+) cells/ field**	**DAPI/ field**	**%GFAP/ DAPI**	**MEAN %**	**GFAP(+) cells/ field**	**DAPI/ field**	**%GFAP/ DAPI**	**MEAN %**
1.1	10	156	6.4		26	66	39.4		10	46	21.7	
1.2	10	128	7.8		22	98	22.4		20	50	40.0	
1.3	18	126	14.3		35	100	35.0		16	40	40.0	
1.4	19	93	20.4	12.2	36	93	38.7	33.9	15	75	20.0	30.4
2.1	15	143	10.5		46	106	43.4		22	55	40.0	
2.2	6	156	3.8		30	110	27.3		47	82	57.3	
2.3	17	131	13.0		35	96	36.5		25	72	34.7	
2.4	2	80	2.5	7.5	30	86	34.9	35.5	39	83	47.0	44.8
3.1	8	106	7.5		32	97	33.0		45	99	45.5	
3.2	13	118	11.0		27	99	27.3		36	84	42.9	
3.3	11	102	10.8		24	97	24.7		30	93	32.3	
3.4	7	77	9.1	9.6	35	76	46.1	32.8	21	45	46.7	41.8
4.1	8	160	5.0		27	88	30.7		29	90	32.2	
4.2	9	121	7.4		26	91	28.6		59	101	58.4	
4.3	8	176	4.5		28	71	39.4		22	66	33.3	
4.4	19	114	16.7	8.4	37	115	32.2	32.7	52	91	57.1	45.3
5.1	10	140	7.1		23	56	41.1		36	67	53.7	
5.2	15	163	9.2		31	68	45.6		19	36	52.8	
5.3	8	110	7.3		30	60	50.0		42	66	63.6	
5.4	8	123	6.5	7.5	41	80	51.3	47.0	17	43	39.5	52.4
6.1	14	140	10.0		23	46	50.0		16	40	40.0	
6.2	9	190	4.7		43	77	55.8		24	65	36.9	
6.3	10	150	6.7		25	47	53.2		28	76	36.8	
6.4	9	143	6.3	6.9	34	59	57.6	54.2	23	58	39.7	38.4
7.1	20	165	12.1		48	77	62.3		21	45	46.7	
7.2	8	178	4.5		51	60	85.0		29	64	45.3	
7.3	6	137	4.4		48	68	70.6		17	42	40.5	
7.4	13	167	7.8	7.2	62	82	75.6	73.4	17	41	41.5	43.5
8.1	18	136	13.2		26	52	50.0		20	42	47.6	
8.2	9	151	6.0		44	73	60.3		19	38	50.0	
8.3	8	89	9.0		36	51	70.6		13	45	28.9	
8.4	8	131	6.1	8.6	32	68	47.1	57.0	27	83	32.5	39.8
9.1	7	36	19.4		53	94	56.4		26	54	48.1	
9.2	11	101	10.9		38	63	60.3		24	47	51.1	
9.3	6	128	4.7		25	75	33.3		23	45	51.1	
9.4	6	50	12.0	11.8	38	97	39.2	47.3	31	42	73.8	56.0
10.1	9	56	16.1		33	59	55.9		26	47	55.3	
10.2	3	56	5.4		39	49	79.6		21	61	34.4	
10.3	3	70	4.3		61	99	61.6		29	67	43.3	
10.4	16	110	14.5	10.1	26	64	40.6	59.4	37	54	68.5	50.4
11.1	6	50	12.0		64	97	66.0		15	38	39.5	
11.2	10	140	7.1		50	98	51.0		16	50	32.0	
11.3	7	53	13.2		54	88	61.4		33	56	58.9	
11.4	7	50	14.0	11.6	67	100	67.0	61.3	16	39	41.0	42.9
12.1	14	60	23.3		51	86	59.3		19	56	33.9	
12.2	10	120	8.3		54	98	55.1		25	68	36.8	
12.3	8	93	8.6		31	90	34.4		28	57	49.1	
12.4	8	103	7.8	12.0	40	88	45.5	48.6	20	41	48.8	42.1
MEAN				**9.4**				**48.6**				**44.0**
SD				**2.0**				**13.1**				**6.8**
SE				**0.6**				**3.8**				**2.0**

12.5.6 CULTURE CHARACTERISATION OF DIFFERENTIATING NEUROSPHERES AFTER 6 DIV (MBP)

	E12				E13				E14			
C.SLIP NO./ FIELD NO.	**MBP(+) cells/** field	**DAPI/** field	**%MBP/** DAPI	**MEAN** %	**MBP(+) cells/** field	**DAPI/** field	**%MBP/** DAPI	**MEAN** %	**MBP(+) cells/** field	**DAPI/** field	**%MBP/** DAPI	**MEAN** %
1.1	4	96	4.2		7	80	8.8		8	121	6.6	
1.2	9	125	7.2		11	73	15.1		8	88	9.1	
1.3	6	94	6.4		6	74	8.1		7	68	10.3	
1.4	3	101	3.0	5.2	1	57	1.8	8.4	5	56	8.9	8.7
2.1	4	106	3.8		8	70	11.4		10	93	10.8	
2.2	8	117	6.8		4	55	7.3		9	87	10.3	
2.3	5	87	5.7		6	103	5.8		10	123	8.1	
2.4	8	130	6.2	5.6	9	72	12.5	9.3	15	130	11.5	10.2
3.1	15	167	9.0		4	62	6.5		5	93	5.4	
3.2	4	101	4.0		6	101	5.9		3	94	3.2	
3.3	4	73	5.5		3	72	4.2		8	96	8.3	
3.4	5	103	4.9	5.8	10	77	13.0	7.4	18	100	18.0	8.7
4.1	5	137	3.6		5	72	6.9		7	85	8.2	
4.2	2	37	5.4		5	110	4.5		5	110	4.5	
4.3	4	77	5.2		3	53	5.7		7	100	7.0	
4.4	4	85	4.7	4.7	4	56	7.1	6.1	5	64	7.8	6.9
5.1	9	230	3.9		3	69	4.3		10	58	17.2	
5.2	6	180	3.3		2	73	2.7		16	86	18.6	
5.3	9	207	4.3		4	85	4.7		12	82	14.6	
5.4	3	130	2.3	3.5	3	107	2.8	3.6	8	55	14.5	16.3
6.1	3	87	3.4		1	87	1.1		17	60	28.3	
6.2	2	95	2.1		2	93	2.2		9	71	12.7	
6.3	4	139	2.9		3	101	3.0		6	25	24.0	
6.4	9	130	6.9	3.8	4	107	3.7	2.5	6	46	13.0	19.5
7.1	7	203	3.4		3	110	2.7		5	38	13.2	
7.2	4	155	2.6		6	96	6.3		11	75	14.7	
7.3	1	100	1.0		4	72	5.6		8	68	11.8	
7.4	2	117	1.7	2.2	1	80	1.3	3.9	5	55	9.1	12.2
8.1	1	70	1.4		3	78	3.8		6	31	19.4	
8.2	11	210	5.2		1	59	1.7		10	60	16.7	
8.3	2	68	2.9		3	81	3.7		12	63	19.0	
8.4	6	86	7.0	4.1	1	31	3.2	3.1	9	48	18.8	18.5
9.1	2	69	2.9		11	85	12.9		7	43	16.3	
9.2	2	78	2.6		6	87	6.9		7	29	24.1	
9.3	1	87	1.1		5	110	4.5		5	27	18.5	
9.4	2	101	2.0	2.1	2	85	2.4	6.7	16	53	30.2	22.3
10.1	2	140	1.4		5	101	5.0		12	57	21.1	
10.2	2	73	2.7		5	72	6.9		11	81	13.6	
10.3	2	86	2.3		4	82	4.9		8	63	12.7	
10.4	3	95	3.2	2.4	3	57	5.3	5.5	6	68	8.8	14.0
11.1	1	70	1.4		7	87	8.0		4	40	10.0	
11.2	2	83	2.4		2	81	2.5		6	38	15.8	
11.3	2	108	1.9		3	80	3.8		5	50	10.0	
11.4	2	60	3.3	2.3	5	72	6.9	5.3	3	40	7.5	10.8
12.1	2	80	2.5		2	89	2.2		2	30	6.7	
12.2	4	82	4.9		3	63	4.8		8	44	18.2	
12.3	1	88	1.1		4	85	4.7		9	65	13.8	
12.4	1	128	0.8	2.3	5	110	4.5	4.1	8	44	18.2	14.2
MEAN				**3.7**				**5.5**				**13.5**
SD				**1.4**				**2.1**				**4.8**
SE				**0.4**				**0.6**				**1.4**

12.5.7 CULTURE CHARACTERISATION OF DIFFERENTIATING NEUROSPHERES AFTER 6 DIV (NESTIN)

	E12				E13				E14			
C.SLIP NO./ FIELD NO.	**NESTIN(+) cells/** field	**DAPI/** field	**%NESTIN/** DAPI	**MEAN** %	**NESTIN(+) cells/** field	**DAPI/** field	**%NESTIN/** DAPI	**MEAN** %	**NESTIN(+) cells/** field	**DAPI/** field	**%NESTIN/** DAPI	**MEAN** %
1.1	34	60	56.7		39	123	31.7		42	76	55.3	
1.2	39	50	78.0		33	104	31.7		40	78	51.3	
1.3	22	36	61.1		37	124	29.8		32	64	50.0	
1.4	36	100	36.0	57.9	39	100	39.0	33.1	41	97	42.3	49.7
2.1	40	65	61.5		38	101	37.6		28	51	54.9	
2.2	50	87	57.5		31	97	32.0		65	80	81.3	
2.3	38	83	45.8		34	95	35.8		13	62	21.0	
2.4	66	120	55.0	54.9	61	141	43.3	37.2	15	51	29.4	46.6
3.1	75	97	77.3		44	117	37.6		40	61	65.6	
3.2	50	85	58.8		37	97	38.1		32	73	43.8	
3.3	80	85	94.1		36	98	36.7		37	78	47.4	
3.4	90	95	94.7	81.2	44	106	41.5	38.5	32	59	54.2	52.8
4.1	100	107	93.5		29	120	24.2		54	85	63.5	
4.2	62	97	63.9		34	108	31.5		15	74	20.3	
4.3	43	61	70.5		37	131	28.2		45	73	61.6	
4.4	40	61	65.6	73.4	25	103	24.3	27.0	23	52	44.2	47.4
5.1	46	86	53.5		46	103	44.7		26	44	59.1	
5.2	27	51	52.9		39	99	39.4		26	51	51.0	
5.3	25	50	50.0		32	84	38.1		14	37	37.8	
5.4	10	36	27.8	46.1	32	81	39.5	40.4	8	30	26.7	43.6
6.1	98	113	86.7		33	83	39.8		15	37	40.5	
6.2	35	71	49.3		35	105	33.3		4	29	13.8	
6.3	17	34	50.0		27	75	36.0		17	35	48.6	
6.4	38	80	47.5	58.4	31	85	36.5	36.4	17	35	48.6	37.9
7.1	30	60	50.0		26	52	50.0		29	51	56.9	
7.2	8	15	53.3		22	64	34.4		18	83	21.7	
7.3	53	107	49.5		22	68	32.4		31	79	39.2	
7.4	25	51	49.0	50.5	18	59	30.5	36.8	19	56	33.9	37.9
8.1	62	82	75.6		37	111	33.3		25	41	61.0	
8.2	65	129	50.4		25	69	36.2		21	42	50.0	
8.3	77	155	49.7		29	84	34.5		22	48	45.8	
8.4	81	110	73.6	62.3	33	89	37.1	35.3	20	40	50.0	51.7
9.1	73	95	76.8		34	59	57.6					
9.2	19	36	52.8		20	78	25.6					
9.3	21	52	40.4		36	76	47.4					
9.4	33	69	47.8	54.5	33	98	33.7	41.1				
10.1	52	75	69.3		54	81	66.7					
10.2	68	93	73.1		49	79	62.0					
10.3	35	64	54.7		44	82	53.7					
10.4	44	70	62.9	65.0	67	101	66.3	62.2				
11.1	62	90	68.9		47	66	71.2					
11.2	69	107	64.5		33	67	49.3					
11.3	37	67	55.2		29	55	52.7					
11.4	32	46	69.6	64.5	54	82	65.9	59.8				
12.1	111	131	84.7		40	86	46.5					
12.2	50	95	52.6		66	97	68.0					
12.3	90	120	75.0		42	101	41.6					
12.4	35	77	45.5	64.5	51	103	49.5	51.4				
MEAN				**61.1**				**41.6**				**46.0**
SD				9.7				10.7				5.7
SE				2.8				3.1				2.0

12.5.8 CULTURE CHARACTERISATION OF DIFFERENTIATING NEUROSPHERES AFTER 6 DIV (TH) E12

C.SLIP NO./ FIELD NO.	**NESTIN(+) cells/** field	**DAPI/** field	**%NESTIN/** DAPI	**MEAN** %
1.1	27	1000	2.7	
1.2	17	584	2.9	
1.3	24	576	4.2	
1.4	5	810	0.6	2.6
2.1	10	512	2.0	
2.2	6	764	0.8	
2.3	23	720	3.2	
2.4	21	613	3.4	2.3
3.1	20	510	3.9	
3.2	5	572	0.9	
3.3	17	820	2.1	
3.4	5	612	0.8	1.9
4.1	4	645	0.6	
4.2	5	616	0.8	
4.3	15	960	1.6	
4.4	2	610	0.3	0.8
5.1	7	573	1.2	
5.2	19	692	2.7	
5.3	14	627	2.2	
5.4	15	726	2.1	2.1
6.1	6	555	1.1	
6.2	12	658	1.8	
6.3	6	539	1.1	
6.4	9	721	1.2	1.3
7.1	5	654	0.8	
7.2	6	711	0.8	
7.3	9	681	1.3	
7.4	12	723	1.7	1.1
8.1	15	653	2.3	
8.2	9	576	1.6	
8.3	4	457	0.9	
8.4	19	650	2.9	1.9
9.1	16	710	2.3	
9.2	10	612	1.6	
9.3	6	519	1.2	
9.4	13	645	2.0	1.8
MEAN				**1.8**
SD				**0.6**
SE				**0.2**

12.6 APPENDIX F (CHAPTER 9)

12.6.1 CULTURE CHARACTERISATION OF E12 VM AFTER 1 DIV

C.SLIP NO./ FIELD NO.	TUJ-1(+) cells/ field	DAPI/ field	%TUJ-1/ DAPI	MEAN %	TH(+) cells/ field	DAPI/ field	%TH/ DAPI	MEAN %	Brd-U(+) cells/ field	BRD-U/ field	%BRD-U/ DAPI	MEAN %
1.1	48	70	68.6		2	81	2.47		16	57	28.1	
1.2	55	82	67.1		2	119	1.68		25	65	38.5	
1.3	41	67	61.2		3	133	2.26		11	49	22.4	
1.4	36	87	41.4	59.6	1	124	0.81	1.80	11	56	19.6	27.2
2.1	28	53	52.8		2	112	1.79		27	71	38.0	
2.2	30	48	62.5		3	114	2.63		26	69	37.7	
2.3	33	54	61.1		3	82	3.66		24	121	19.8	
2.4	46	85	54.1	57.6	2	149	1.34	2.35	48	149	32.2	31.9
3.1	36	58	62.1		2	120	1.67		19	83	22.9	
3.2	48	82	58.5		2	130	1.54		14	72	19.4	
3.3	47	78	60.3		5	145	3.45		18	57	31.6	
3.4	53	91	58.2	59.8	1	101	0.99	1.91	11	58	19.0	23.2
4.1	44	86	51.2		1	105	0.95		17	53	32.1	
4.2	46	70	65.7		2	134	1.49		14	72	19.4	
4.3	42	69	60.9		4	142	2.82		27	102	26.5	
4.4	39	60	65.0	60.7	2	84	2.38	1.91	17	46	37.0	28.7
5.1	29	140	20.7		4	121	3.31		21	87	24.1	
5.2	45	96	46.9		3	97	3.09		13	90	14.4	
5.3	63	109	57.8		1	133	0.75		21	82	25.6	
5.4	61	92	66.3	47.9	2	129	1.55	2.18	20	56	35.7	25.0
6.1	30	60	50.0		2	88	2.27		15	47	31.9	
6.2	50	85	58.8		2	96	2.08		26	111	23.4	
6.3	43	80	53.8		1	131	0.76		13	62	21.0	
6.4	45	88	51.1	53.4	4	157	2.55	1.92	20	91	22.0	24.6
7.1	40	72	55.6		3	114	2.63					
7.2	48	90	53.3		2	164	1.22					
7.3	61	112	54.5		2	131	1.53					
7.4	44	89	49.4	53.2	3	129	2.33	1.93				
8.1	70	112	62.5		1	145	0.69					
8.2	59	120	49.2		2	131	1.53					
8.3	41	87	47.1		4	179	2.23					
8.4	60	91	65.9	56.2	3	121	2.48	1.73				
MEAN				**56.0**				**1.97**				**26.8**
SD				**4.3**				**0.20**				**3.2**
SE				**1.5**				**0.07**				**1.3**

12.6.2 COUNTS OF TH-POSITIVE CELLS IN E12VM CULTURES AFTER TREATMENT WITH GDF-5 FOR 1 DIV

	Control	10ng/ml GDF-5		
c.slip no. / field no.	**TH (+) cells/field**	**TH (+) cells/field**	**Average/field**	**Average/field**
1.1	3	9		
1.2	3	3		
1.3	6	6		
1.4	7	5	4.8	5.8
2.1	2	3		
2.2	2	9		
2.3	2	8		
2.4	5	9	2.8	7.3
3.1	5	7		
3.2	10	10		
3.3	1	8		
3.4	2	9	4.5	8.5
4.1	4	8		
4.2	2	8		
4.3	2	12		
4.4	4	6	3.0	8.5
5.1	7	10		
5.2	9	13		
5.3	10	33		
5.4	14	26	10.0	20.5
6.1	3	23		
6.2	5	11		
6.3	10	10		
6.4	15	15	8.3	14.8
7.1	9	9		
7.2	12	8		
7.3	8	7		
7.4	2	10	7.8	8.5
8.1	16	12		
8.2	10	15		
8.3	7	9		
8.4	9	11	10.5	11.8
9.1	7	9		
9.2	7	4		
9.3	4	9		
9.4	4	9	5.5	7.8
MEAN			**6.3**	**10.4**
SD			**2.9**	**4.6**
SE			**1.0**	**1.5**

12.6.3 COUNTS OF APOPTOTIC CELLS IN E12VM CULTURES AFTER TREATMENT WITH GDF-5 FOR 1 DIV

	Control	10ng/ml GDF-5		
c.slip no. / field no.	**Apoptotic cells/field**	**Apoptotic cells/field**	**Average/field**	**Average/field**
1.1	7	20		
1.2	30	8		
1.3	20	20		
1.4	3	11	15.0	14.8
2.1	3	11		
2.2	5	16		
2.3	16	5		
2.4	15	3	9.8	8.8
3.1	20	6		
3.2	9	14		
3.3	6	8		
3.4	6	6	10.3	8.5
4.1	25	12		
4.2	13	20		
4.3	14	16		
4.4	26	14	19.5	15.5
5.1	5	3		
5.2	12	2		
5.3	2	15		
5.4	3	7	5.5	6.8
6.1	12	9		
6.2	7	10		
6.3	5	20		
6.4	9	8	8.3	11.8
7.1	15	10		
7.2	5	9		
7.3	11	8		
7.4	23	10	13.5	9.3
8.1	4	16		
8.2	15	8		
8.3	10	12		
8.4	8	12	9.3	12.0
9.1	12	19		
9.2	12	12		
9.3	8	12		
9.4	4	8	9.0	12.8
MEAN			**11.1**	**11.1**
SD			**4.2**	**3.0**
SE			**1.4**	**1.0**

12.6.4 COUNTS OF TOTAL CELLS IN E12VM CULTURES AFTER TREATMENT WITH GDF-5 FOR 1 DIV

	Control	10ng/ml GDF-5		
c.slip no. / field no.	**Total cells/field**	**Total cells/field**	**Average/field**	**Average/field**
1.1	57	42		
1.2	65	65		
1.3	49	61		
1.4	56	50	56.8	54.5
2.1	71	43		
2.2	69	76		
2.3	121	85		
2.4	149	85	102.5	72.3
3.1	83	96		
3.2	72	121		
3.3	57	26		
3.4	58	93	67.5	84.0
4.1	53	84		
4.2	72	90		
4.3	102	50		
4.4	46	47	68.3	67.8
5.1	87	96		
5.2	90	113		
5.3	82	54		
5.4	56	86	78.8	87.3
6.1	47	101		
6.2	111	110		
6.3	62	73		
6.4	91	90	77.8	93.5
MEAN			**75.3**	**76.5**
SD			**15.6**	**14.4**
SE			**6.4**	**5.9**

12.6.5 COUNTS OF BrdU-POSITIVE CELLS IN E12VM CULTURES AFTER TREATMENT WITH GDF-5 FOR 1 DIV

	Control	10ng/ml GDF-5		
c.slip no. / field no.	**BrdU(+) cells/field**	**BrdU(+) cells/field**	**Average/field**	**Average/field**
1.1	16	8		
1.2	25	17		
1.3	11	24		
1.4	11	13	15.8	15.5
2.1	27	8		
2.2	26	23		

2.3	24	22		
2.4	48	22	31.3	18.8
3.1	19	19		
3.2	14	18		
3.3	18	6		
3.4	11	21	15.5	16.0
4.1	17	13		
4.2	14	31		
4.3	27	20		
4.4	17	12	18.8	19.0
5.1	21	18		
5.2	13	33		
5.3	21	16		
5.4	20	20	18.8	21.8
6.1	15	24		
6.2	26	16		
6.3	13	19		
6.4	20	19	18.5	19.5
MEAN			**19.8**	**18.4**
SD			**5.8**	**2.3**
SE			**2.4**	**1.0**

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