Neural Stem Cells for Cell Replacement Therapy in Huntington's Disease.

A dissertation for the degree of Doctor of Philosophy

at

Cardiff University

by

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Summary

The research reported in this thesis focused on the potential of neural precursor cells to provide a suitable source of neurones which can be used in cell replacement strategies for Huntington's disease. Specifically, the parameters affecting the differentiation of these cells into neuronal phenotypes were addressed and increasing the survival of proliferating and differentiating neurones was attempted. *In vivo* characteristics and the fibre projections of primary and 10 day expanded ENPs was also assessed. The limitations of xenografts in this thesis led to the search for an alternative model system for such experiments.

Chapter Three involved an extensive study investigating the effects of a range of concentrations of FGF-2 and EGF on the proliferation and more importantly the neuronal differentiation of murine ENPs over 6 passages in culture, and it was found that the concentration had an effect on the neuronal proportion as well as the neuronal yield of these cultures.

Chapter 4 examined the turnover of neuronal precursors in the ENP population cultured in the presence of FGF2 and EGF, using BrdU. The ongoing proliferation of neuronal precursors within ENP cultures was observed and the addition of the growth factors: CNTF, BDNF, HGF and NGF, to enhance the survival of these neurons on differentiation had no effect.

Chapter 5 examined the potential of 10 day expanded human striatal ENPs to maintain a striatal like phenotype both *in vitro* and *in vivo* in comparison to primary foetal tissue. In vitro after 10 days expansion ENPs differentiated into DARPP-32 positive neurons and this characteristic was maintained *in vivo,* in a lesion model of HD, albeit to a much lesser extent. This study was limited by the need for ongoing immunosuppression which reduced the life span of the host animal.

Chapter 6 investigates further the potential of ENPs. The ability for these cells to send long projections in the host brain and therefore repairing the circuitry lost or damaged as a result of the disease. A four way analysis was carried out examining both allo- and xenograft environments with both primary and 10 day expanded ENPs. Mouse grafts were used to address the allograft environment given that such an experiment is not possible with human tissue and both human and mouse tissue addressed the xenograft environment. To overcome the issues associated with labelling the grafted tissue in the host brain, several techniques were employed, including; the use of the GFP transgenic mouse, lentiviral labelling of the cells with the LacZ gene and iontophoretic labelling of the graft with anterograde tracers. ENP grafts were shown to send out longer projections than that of primary tissue although this may be due to migration of the grafted cells.

Chapter 7 addresses the issue of immunosuppression of xenografted animals. An alternative model system was explored with the hypothesis being that it would be possible to tolerise the animal in the neonatal period to the xenograft tissue that would subsequently be used for intrastriatal grafting in the adult. Indeed, tolerising the animal resulted in healthy surviving grafts in the adult without the need for daily immunosuppression.

Work presented in this thesis contributes some understanding to the biology of neural stem cells and neural xenografts that may ultimately be used for neural transplantation therapies in HD.

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Publications

Work presented in this thesis has formed part of the following papers that have been published, or are currently submitted for publication.

Chapter 1

Kelly CM, Rosser AE & Dunnett SB. Neuronal cells for transplantation in Huntington's disease. In Sanberg PR and Davis CD (eds), *Cell Therapy for Brain Repair*, Humana Press, Totowa, New Jersey, *in press*

Chapter 3

Kelly CM, Zietlow R, Dunnett SB, Rosser AE (2003) The effects of various concentrations of FGF-2 on the proliferation and neuronal yield of murine embryonic neural precursors *in vitro*. Cell Transplantation, 12: 215-223

Kelly CM, Tyers P, ter Borg M, Svendsen CN, Dunnett SB, Rosser AE (2005) EGF and FGF- 2 responsiveness of rodent neural precursors derived from the embryonic CNS. Brain Research Bulletin, *in press*

Abbreviations

CAPIT	Core Assessment Protocol for Intracerebral Transplantation
CNS	Central nervous system
Cpu	Caudate-Putamen
CRL	Crown Rump Length
CsA	Cyclosporin A
CTX	Cortex
DARPP-32	Dopamine and adenosine 3'5'-monophosphate regulated phosphprotein
DMEM	Dulbecco's Modified Eagle's Medium
DNase	Deoxyribonuclease
E	Embryonic day
EGF	Epidermal growth factor
ES	Embryonic stem cell
FGF	Fibroblast growth factor
GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GPe	Globus Pallidus (external segment)
GPi	Globus Pallidus (internal segment)
HBSS	Hanks buffered saline solution
HD	Huntington's disease
LGE	Lateral ganglionic eminence
LIF	Leukaemia inhibitory factor
MFB	Medial forebrain bundle
MGE	Medial ganglionic eminence
NGS	Normal goat serum
NK	Neuman Keuls statistical test
P	Passage
PBS	Phosphate buffered saline
PD	Parkinson's disease
S.E.M.	Standard error of the mean
SN	Substantia nigra
STN	Subthalamic nucleus

TBS	TRIS buffered saline
TH	Tyrosine hydroxylase
TNS	TRIS non-saline
TRIS	Trizma base
VM	Ventral mesencephalon
VTA	Ventral tegmental area
WGE	Whole ganglionic eminence

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Chapter One

Introduction

1.1 Huntington's Disease

George Huntington described the disease that took his name in his now well-known paper of 1872 (Huntington, 1872). Huntington's disease (HD) is a progressive and devastating neurodegenerative disorder that affects approximately 5-10 per 100,000 in the caucasian community (Harper, 1996). It is inherited in an autosomal, dominant fashion, and can be reliably diagnosed by means of an accurate DNA test. A number of triplet repeat disorders have now been described, and these fall into two groups. In the first, the triplet repeat falls within the coding regions of the gene. HD belongs in this group and in all but one of these disorders, the triplet repeat is a CAG repeat sequence, coding for a sequence of glutamine residues. The other diseases identified to date are the spinocerebellar ataxias (SCAs) 1, 2, 3, 6, 7 and 17, spinal and bulbar muscular atrophy (SBMA) and dentatorubral pallidoluysian atrophy (DRPLA). In the second group, the repeat falls within the non-coding regions, and these include Fragile X Syndrome, Myotonic Dystrophy and Friedreich's Ataxia (Timchenko and Caskey, 1999; Reddy et al., 1999).

The clinical symptoms of HD include a movement disorder (predominantly, chorea, bradykinesia, rigidity and dystonia), as well as a number of other symptoms including dysphasia and dysarthria, intellectual impairment (frontal in nature, at least in the early stages), and psychiatric disturbance. Although by the mid to late stages of the

disease most patients have some symptoms across all categories, the predominant symptoms in the early stage vary from patient to patient. Furthermore, although disease onset is usually defined by the appearance of motor disorders, subtle cognitive decline may precede the onset of motor symptoms by up to 10 years (Lawrence et al., 1998). The disease most commonly manifests in the 3rd and 4th decades and is relentlessly progressive with death approximately 20 years after diagnosis (Naarding et al., 2001; Ross and Margolis, 2001; Masino and Pastore, 2001). At the time of writing, symptomatic treatments are limited and there are no proven disease-modifying therapies available.

1.2 Genetics of HD

The gene for HD, now known as *huntingtin* (*htt*), comprises 67 exons (Ho et al., 2001) and is found on the end of chromosome 4 (4p16.3) (Huntington's Disease Collaborative Research Group, 1993). The gene contains in exon 1 a repeated triplet CAG sequence that encodes for the amino acid glutamine, which is the region that is expanded in the mutant gene. The *htt* gene codes for the 350Kda protein Huntingtin (Htt), the normal function of which is still unclear. In the normal gene there are fewer than 36 triplet repeats, although those with repeats of 35-38 triplets are currently considered to be intermediate, and the extent to which any phenotype results from repeats in this range remains to be determined (Georgiou-Karistianis et al., 2003). There is a correlation between repeat length and age of disease onset, although this is largely due to the early onset in patients with very large repeat numbers, whereas for the majority with repeat numbers in the 40s and low 50s the correlation is poor.

1.3 Pathology of HD

There have been considerable advances in understanding the cellular pathology of HD over the last 5-10 years, although the precise mechanism by which the gene induces cell death remains elusive. It appears that mutant Htt produces its effect in a 'gain of function' manner, although loss of function may also play a role (Cattaneo and Calabresi, 2002). Evidence in support of gain of function has been derived from the observation that exogenous mutant Htt can cause degeneration in a variety of cell culture systems despite the presence of endogenous wild-type Htt and small fragments have been shown to be more toxic than larger ones. There is accumulating evidence that aggregation of the mutant protein plays a key role, producing micro-aggregates as well as larger cellular aggregates which are now thought of as pathological hallmarks of the disease (Wanker, 2000). In vivo, post mortem antibody staining suggests that intraneuronal aggregates are predominantly comprised of truncated N-terminal fragments rather than full length mutant Htt (Furtado et al., 1996). The aggregates are believed to be the result of mis-folded expanded polyglutamine repeat sequences, leading to impaired cellular metabolism and cell death (Li and Li, 2004). Whether intracellular aggregates are part of the pathogenic process, for example by interfering with cellular trafficking (Saudou et al., 1998), are beneficial to the cell (Arrasate et al., 2004), or are simply a disease marker with no interfering or beneficial effects is still controversial. It seems that wild-type huntingtin may be important in a wide range of cellular processes, and a number of potential effects of the mutant protein have been postulated as being of potential relevance to the disease state. For example: the cAMP response element (CRE)-mediated pathway shows the early disruption and is significantly down-regulated when compared to the retinoic acid response element (RARE) and the nuclear factor-κ B pathways (Sugars et al., 2004), suggesting that

reduced CRE dependent transcription may contribute to disease pathogenesis; polyglutamine expansion may also trigger apoptosis via the activation of the caspase 1 and caspase 8 (Li et al., 2000) pathways; and mutant Htt seems to interfere with proteasome function, thus preventing toxic protein fragments from being removed from the cell (Venkatraman et al., 2004; Bence et al., 2001). A truly rational approach to the development of a disease modifying-therapy will require a more complete understanding of these processes.

At a macroscopic level, the pathology of HD is characterised by neuronal loss in the head of the caudate and putamen of the striatum (Figure 1.1), with the medium spiny projection neurones being more affected than striatal interneurones (Ross and Margolis, 2001). As a result of the neuronal loss, there is eventually significant atrophy of striatal structures, with a compensatory expansion of the lateral ventricles. PET scans have shown there to be a progressive loss of D2 binding in the HD brain (Andrews et al., 1999). The remaining striatum is hypometabolic, with energy production and oxidative metabolism being significantly reduced (Kopyov et al., 1998). As the disease progresses, the pathology becomes more widespread, including wide areas of neocortex, to the point where overall brain weight may decrease by up to 25-30%. Gliosis is seen alongside the marked neuronal loss. Neuronal loss in the cortex is found to be layer specific, with the greatest loss seen in layer VI and significant further loss seen in layers III and V (Reddy et al., 1999; Ross and Margolis, 2001).

Figure 1.1

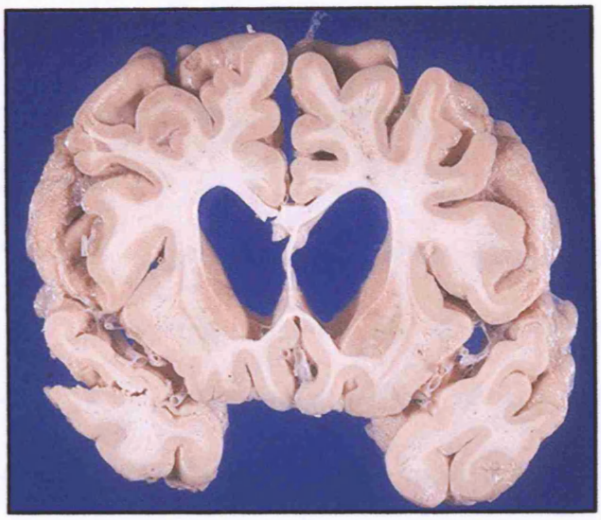


Figure 1.1 Coronal section through HD brain. Note the degree of atrophy of the caudate nuclei and the cerebral cortex. Courtesy of Prof. JR Hodges

1.4 The developing striatum

Development of the vertebrate nervous system begins with neural induction, when a dorsal region of the embryonic ectoderm becomes specified as neural plate. Neurulation then occurs, when the neural plate folds in on itself, forming the neural tube which is lined by a pseudostratified columnar epithelium consisting of uncommitted progenitors from which the future CNS will arise (Jain et al., 2001; Kandel et al., 2001). The adult human striatum consists of the caudate nucleus and the putamen. The origins of the striatum lie in the ventral telencephalic vesicle of the embryonic brain, derived from an area between the medial septum, and the border of the dorsal telencephalon that later develops into cortex.

In mammalian development, the pallidum and striatum are derived from the transient swellings in the ventral region of the embryonic telencephalon known as the Medial Ganglionic Eminence (MGE), and the Lateral ganglionic Eminence (LGE) (also see Figure 1.4) and the caudal area where they both join, the Caudal Ganglionic Eminence (CGE). The MGE appears first, followed later by the LGE. These areas are collectively known as the Whole Ganglionic Eminence (WGE).

The basal ganglia are comprised of a number of interconnected nuclei linking the basal ganglia to the cortex, the thalamus and the brainstem through several independent pathways. These nuclei are the striatum, the sub thalamic nuclei (STN), the internal and external segments of the globus pallidus (GP*i/e*) and the substantia nigra *pars compacta* and *pars reticulata* (SNc and SNr).

Grafts comprised of tissue dissected from both the medial and lateral ganglionic eminences are heterogenous in composition, containing characteristic patches of striatal like tissue, termed P (patch) zones and tissue that lacks the characteristics of striatal neuronal phenotypes - NP (non patch) zones (Figure 1.2). P zones comprise neurons that are immunopositive for the striatal markers such as acetylcholinesterase (AChE), and DARPP-32 as well as GABA, enkephalin, dynorphin, and substance P. In contrast NP zone neurons are DARPP-32 and AChE negative and it has been suggested that these neurons are in fact not striatal, but have been derived from other progenitor cells and neuroblasts that have been dissected along with the ganglionic eminences (or which are migrating through the GE at this developmental age) and may include neurons from the globus pallidus, amygdala and cortex (Graybiel et al.,

1989a; Zhou et al., 1989; DiFiglia et al., 1988; Walker et al., 1987; Sirinathsinghji et al., 1993).

The proportion of P zones in a striatal graft is influenced by the dissection of the ganglionic eminences. Grafts of tissue from whole GE (both the LGE and MGE) have 30-50% P zones as a proportion of the entire graft volume, whereas grafts of LGE alone resulted in an increase of the proportion of the P zone to 80-90%. Grafts of MGE result in grafts with only 25% P zone (Deacon et al., 1994; Grasbon-Frodl et al., 1996; Graybiel et al., 1989a; Isacson et al., 1985; Olsson et al., 1995; Pakzaban et al., 1993; Watts et al., 2000b; Wictorin et al., 1989b; Fricker-Gates et al., 2001; Grasbon-Frodl et al., 1997).

Figure 1.2

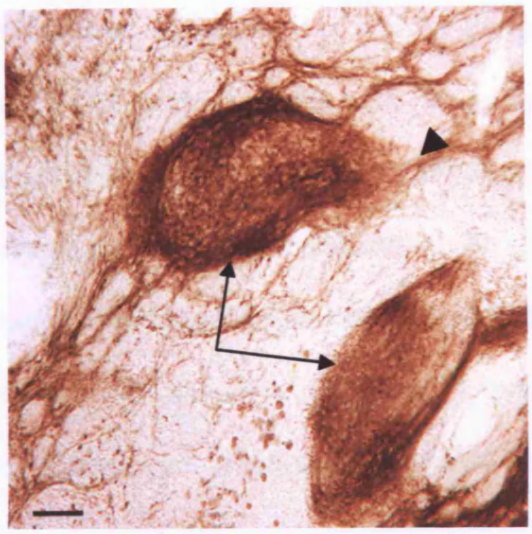


Figure 1.2 The P (arrows) and NP (arrow head) zones of the striatum, stained with AChE.

1.4.1 Molecular aspects of striatal development

A host of molecular changes occur throughout development in response to fluctuations in the cells internal and external environment. Some of these changes regulate the expression of genes that code for many types of small molecules, some of which will act on the cell to influence its development and differentiation. These molecules range from signalling proteins such as Delta and Notch (Grandbarbe et al., 2003) that inhibit neuronal fate and promote glial cell fate, later promoting astrocytic differentiation, to the newly discovered potential of the post transcriptional control exerted by other elements such as gene silencing using RNA interference molecules

(Fire et al., 1998). All of these changes are controlled through changes in the transcription of genes, and the way the gene products interact with each other.

Sonic hedgehog" (Shh) is a morphogen that has a variety of effects in the developing embryo. Deletion of this gene shows that it is vital for the generation of the globus pallidus and the striatum (Kohtz et al., 1998), with the telencephalon being greatly dysmorphic and reduced in size (Machold et al., 2003) as well as changes in many other ventral structures along the rostra-caudal axis of the CNS. It induces ventral identity in a concentration-dependant manner (Shimamura and Rubenstein, 1997) and has been shown to induce the expression of Nkx-2.1 a brain marker in the MGE. Although Nkx-2.1 and Shh are both expressed in the MGE, neither has been detected in the LGE. However, after targeted mutagenesis of Shh the LGE fails to form as expression of the marker Dlx-5 is lost and the neocortical marker Emx-1 expands ventrally to take its place (Kohtz et al., 1998). Also, at a later stage of development (around E8.5 to E9.5 in the mouse), Shh expression has been shown to induce the expression of LGE proteins (Kohtz et al., 1998) and it seems that it is these changes within the ganglionic eminence, that regulate the formation of firstly the MGE, and then later the LGE (Kohtz et al., 1998).

Four of the Dlx genes (Dlx 1/2/5 and 6 (Anderson et al., 1997b; Eisenstat et al., 1999)) are expressed in the developing ganglionic eminences and have been described as being critical for striatal development (Jain et al., 2001; Rubenstein and Shimamura, 1998; Panganban and Rubenstein, 2002; Marin et al., 2000; Eisenstat et al., 1999; Yun et al., 2002; Corbin et al., 2000; Nery et al., 2002; Rallu et al., 2002).

These Dlx genes are thought to control the differentiation of a subset of GABA-ergic neurons of the basal ganglia and cerebral cortex (Panganiban and Rubenstein, 2002b).

The expression of Dlx-1 and Dlx-2 were originally thought to be indistinguishable, although recent work has indicated that Dlx-2 expression precedes that of Dlx-1, which itself precedes expression of Dlx-5, although early born striatal cells still express Dlx-5 and Dlx-6, indicating that these earlier born cells express Dlx-5/6 independently of Dlx-1/2 (Eisenstat et al., 1999). Single mutants for Dlx-1, Dlx-2 and Dlx-5 have shown no discernable forebrain defects, suggesting there is some degree of genetic redundancy.

However, the Dlx-1/2 double mutant shows an absence of the later-born (after E12.5 (Eisenstat et al., 1999) GABA-ergic interneurons containing somatostatin, neuropeptide-Y and nitric oxide (Jain et al., 2001). These later born striatal matrix neurons are believed to be derived from the sub ventricular zone (SVZ) of the developing striatum and are thought to remain in the SVZ apparently due to an arrest in their migration (Eisenstat et al., 1999; Anderson et al., 1997d). Tangential migration from the MGE to the cortex and pallidum is also decreased or absent (Anderson et al., 1997c; Anderson et al., 1997a). It seems that Dlx genes in general have a role to play in the development of GABA-ergic neurons, as the expression of the Dlx genes coincides with the location of virtually all neurons that use GABA as their transmitter. Further proof of this is shown in studies where ectopic expression of Dlx genes has induced a GABA-ergic phenotype (Panganiban and Rubenstein, 2002a).

Gsh-2 also leads to the expression of Mash-1 a gene that is closely related to the Drosophila *"achaete-scute"* gene and implicated in striatal early-born cholinergic interneuron development (Jain et al., 2001) in the MGE; a loss of Gsh-2 will cause a subsequent loss of Mash-1, affecting the generation of neural precursors in the MGE by E12.5 (Casarosa et al., 1999) and altering the timing of production of SVZ precursors in the LGE. This leads to a perturbed generation of neuronal populations in the basal ganglia and cortex (as a proportion of cells born in the ganglionic eminence are destined to migrate tangentially towards dorsal regions to become cortex later in development (Casarosa et al., 1999; Nery et al., 2002). Mash-1 is confined to a subpopulation of neural precursor cells. Expression also precedes and ends upon neural differentiation in a similar fashion to its Drosophila homologue (Lo et al., 1991).

Pax-6 is a homeobox gene expressed in the dorsal telencephalon, a region that later develops into cortex, but not in the ventral telencephalon which later forms the striatum (Toresson et al., 2000b). Conversely, Gsh-2, is expressed in the MGE (this is thought to be a result of the presence of Shh along the ventral antero-posterior axis) firstly at around E10.5 (Corbin et al., 2000), then spreading to the WGE later on, but not in the dorsal telencephalon. These two DNA regulating genes (and perhaps many more like them) probably regulate the expression of a further subset of genes that will cause the cortex or striatum to develop a certain fate (Toresson et al., 2000b).

It can be seen, that both Pax-6, Gsh-2 and a number of other genes that are expressed are implicated in dorsal/ventral identity (Corbin et al., 2000; Toresson et al., 2000b; Yun et al., 2001). It seems they take part in laying a 'molecular border line' down

between the ventral ganglionic eminence and the dorsal cortex, as well as many other roles that they may play in cellular differentiation, development and normal activity.

Another gene that is expressed in the foetus as early in development as the 3rd and 4th somite stages and in the MGE at around E9-E9.5 (Yun et al., 2003) is Nkx-2.1. As stated above, this gene is thought to be induced by the presence of Shh (Rubenstein and Shimamura, 1998) along the rostro-ventral axis of the neural tube at around E8.5. Nkx-2.1 is expressed in cells that originate in the MGE but later migrate dorsally into the LGE (although not as far as the cortex (Chapouton et al., 1999). At E14.5 and later stages, Nkx2.1 expression is prominent in the developing globus pallidus and as development continues it's expression can be detected in several other ventral telencephalic structures including the Bed Nucleus of the Stria Terminalis (BNST), parts of the septum, the ventral pallidum and parts of the amygdala (Sussel et al., 1999).

Foxg-1, a gene formerly known as Bf-1 is a winged helix transcriptional factor first expressed at around E8.5-9 (Jain et al., 2001), that acts as a negative regulator in the telencephalon. A lack of expression of Foxg-1 as seen in the mutant Foxg-$1^{-/-}$, results in cortical hypoplasia and still birth (these mutants are only viable up to the age of E18.5 (Hanashima et al., 2004a)). This is thought to be due to progenitors differentiating early leading to reduction of the progenitor population and therefore a reduction in the final numbers of cells within the telencephalon (Xuan et al., 1995). In a recent study Foxg-1 was found to suppress the earliest born cortical neurons (the Cajal/Retzius neurons of layer 1 of the cortex) in normal development, while in the

Foxg-1$^{-/-}$ mouse there is an excess of these cells within the cortex (Hanashima et al., 2004b).

Differentiation of striatal cells may also be associated with the expression of the transcription factor Ebf-1 which is expressed in both the LGE and MGE between E11 and E17.5 (Jain et al., 2001). In the normal brain, antibodies raised against Ebf-1 will highlight later born matrix neurons (Corbin et al., 2000). Although the knockout model shows no defects within the striatum until E17.5, inactivation of this protein does affect cells within the SVZ/mantle transition in the LGE, affecting the differentiation processes coincident with the migration of cells to the mantle zone, therefore leading to abnormal gene expression within the mantle later in development. Due to this, cell death later on in embryological development is increased (Garel et al., 1999).

The Meis family of homeobox genes belong to a super-group of transcription factors that have a Three-Amino acid Loop Extension (TALE). Three Meis genes have been found in vertebrates; Meis-1/2/3. However, only Meis-1 and Meis-2 have been detected in significant levels within the telencephalon; at around E10.5 Meis-1 is detected in low levels in the ventricular zone (VZ) of the ventro-lateral telencephalon while Meis-2 is present in high levels in the areas destined to become the LGE. Twenty four hours later at around E11.5, when the MGE and LGE become morphologically distinct Meis-1 is highly expressed in the CGE and developing amygdala and at lower levels in the LGE and MGE. This protein continues to be expressed in ventro-lateral regions of the striatum, cortex, ventral pallidum and medial septum (Toresson et al., 2000a). In vertebrates Meis-1 and 2 are known to act as co-factors with the *"extra-denticle"* TALE protein homologues Pbx-3 (Toresson et al.,

2000a) and Pbx1 (Knoepfler et al., 1997; Swift et al., 1998). Toresson and colleagues have also shown that Dlx expression overlaps that of Meis and Pbx proteins within the telencephalon.

The active metabolite of vitamin A; Retinoic Acid is another morphogenic molecule that has been implicated in the differentiation of striatal neurons. This molecule works as another gene regulator, via ligand activated transcription factors known as retinoic acid α receptors (RARs) and retinoid acid χ receptors (RXRs) which are both nuclear receptors belonging to the steroid and thyroid hormone receptor family (Zetterstrom et al., 1999).

In the spinal cord, Retinoic acid acts in opposition to Fibroblast Growth Factor (FGF), as a requirement for neuronal differentiation (Diez del Corral et al., 2003). Retinoic acid begins to appear in the developing CNS around E7, and Cellular Retinoic Acid Binding Protein (CRABP) assays have been detected in high amounts in the striatum in newborn rats (when compared to cerebellum, hippocampus and control samples). This expression can also be related to the areas of dopamine D2 receptor innervation within the striatum suggesting a functional relationship between these two molecules. Expression falls back to just above background levels by the time the rat pup reaches 5 weeks old (Zetterstrom et al., 1999). The appearance of retinoic acid is further confirmed by the presence of an enzyme that oxidises retinol (vitamin A) to retinoic acid; aldehyde dehydrogenase-2 (AHD-2, also known as retinaldehyde dehydrogenase) which has also been found within dopaminergic nerve fibre terminals within the striatum, suggesting that retinoic acid may be involved in gene regulation within this area (Zetterstrom et al., 1999) and projection neurons that express GABA,

dynorphin, substance P and enkephalin have also been shown to express RARs and RXRs (Ferre et al., 1997). Retinoic acid has limited or no effect on more ventrally-placed cells due to the opposing action of FGF, hence ventral cells remain under the influence of ventral transcription factors such as Nkx 2.1 (Marklund et al., 2004).

During embryonic development of the striatum, glial cells (possibly radial glia) within the LGE are a localised source of retinoids, allowing cells which are migrating through the LGE into the developing striatum to come under the morphogenic influence of retinoids that could affect their differentiation. Findings that treatment of LGE cells with either RAR or RXR agonists also enhances the striatal neuronal characteristics of these cells further supports this hypothesis (Toresson et al., 1999).

Such studies will hopefully identify genes that may be used *in vitro* and *in vivo* to direct the differentiation of various cells types down a striatal specific neuronal lineage for their subsequent use as a cell source for neural transplantation.

1.5 Neural Transplantation of Foetal Striatal Tissue

The relatively focal loss of medium spiny GABAergic projection neurons in the striatum presents an opportunity to explore neural transplantation as a strategy for cell replacement and circuit reconstruction. The medium spiny neurons of the caudate nucleus and putamen form part of a complex circuitry comprising parallel feedback loops involving discrete areas of cortex and subcortical structures. The medium spiny neurons receive major inputs from the cerebral cortex, thalamus and substantia nigra pars compacta, and have their primary outputs via GABAergic projections to the

globus pallidus and the substantia nigra pars reticulata. Experimental studies in animals over two decades have established that striatal neurons lost through a lesion can be functionally replaced by transplantation of the homologous population of fetal neurons (see below). To achieve this, the developing fetal striatum is dissected, dissociated using enzymatic digestion of the tissue or diced into small tissue pieces less than $1mm^3$, and transplanted stereotaxically into the striatum (see Figure 1.3). Following transplantation the cells continue developing, innervate the surrounding neuropil, and repair the circuitry that has been damaged due to disease.

Figure 1.3

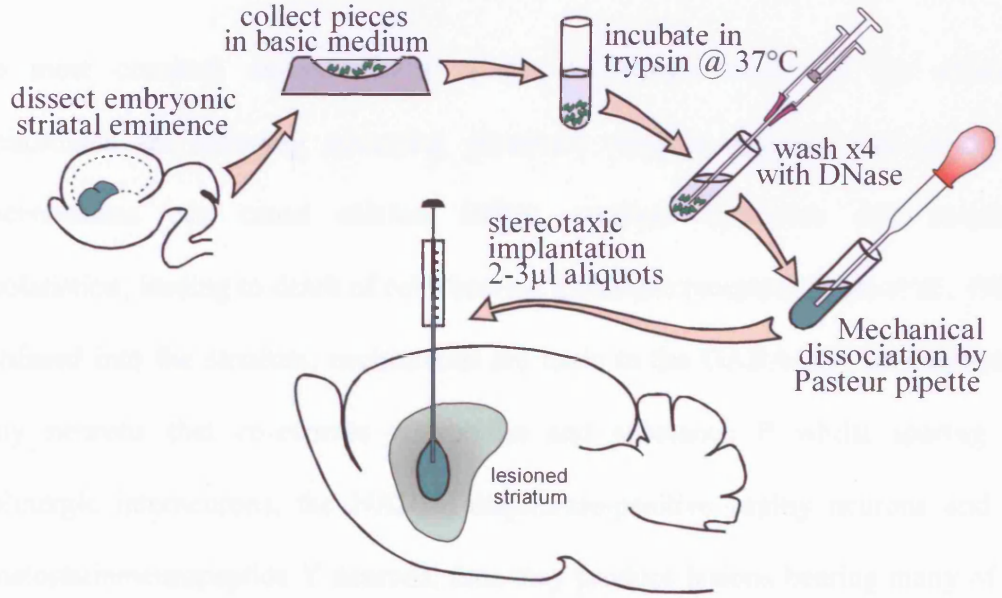


Figure 1.3 Schematic illustration of the dissection, preparation and implantation of neural fetal striatal cells into the lesioned striatum of adult rats.

1.5.1 Animal models

For better understanding of the potential of alternative cell therapies in HD it is important to use an animal model that closely resembles the disease. Metabolic toxins, such as 3-nitropropanoic acid (3-NP), have been investigated for their potential to mimic the human disease. 3-NP inhibits the mitochondrial enzyme succinate dehydrogenase and tricarboxylic acid cycle thus impairing energy metabolism (Coles et al., 1979). Unfortunately there is a large degree of variability when using this toxin in animals thus making it an unattractive model when consistency within and between groups of animals is required, as is the case for behavioural analysis (Lee and Chang, 2004; Borlongan et al., 1997; Brouillet et al., 1995; Ryu et al., 2004; Brouillet et al., 1999).

The most common animal model is the excitotoxic lesion of the striatum. Excitotoxins are naturally occurring glutamate receptor agonists that at certain concentrations can cause calcium influx, protease activation and sustained depolaristion, leading to death of cells bearing glutamate receptors (Beal et al., 1986). If infused into the striatum, excitotoxins are toxic to the GABAergic medium-sized spiny neurons that co-express enkephalin and substance P whilst sparing the cholinergic interneurons, the NADPH-diaphorase-positive aspiny neurons and the somatostatin/neuropeptide Y neurons, thus they produce lesions bearing many of the histological characteristics of the lesions seen in HD. Indeed excitotoxicity is thought to contribute to the cell death in HD, providing further credence for the use of this model of the disease (Ho et al., 2001; Li and Li, 2004; Petersén et al., 1999; Ross and Margolis, 2001; Browne and Beal, 2004). Common excitotoxins used to model HD include kainic acid, ibotenic acid and quinolinic acid. Kainic and ibotenic acid have

been used extensively in the past as they produce large striatal lesions whilst at the same time sparing the fibres of passage; however the neuronal death observed tends to be non-specific (McGeer et al., 1978; Isacson et al., 1985). Behavioural studies using kainic and ibotenic acid lesions have been studied extensively due to the reliable functional deficits with these lesions. Quinolinic acid, which acts specifically at the N-methyl-D-aspartate (NMDA) glutamate receptor, is thought to be more reliable at mimicking the pattern of cell death and gliosis seen in HD (Beal et al., 1986; Beal et al., 1991; Ferrante et al., 1993; Huang et al., 1995; Roberts et al., 1993; Bazzett et al., 1993). Typically, all neuronal types are lost at the centre of the lesion with the more selective cell loss being observed towards the periphery of the lesion. Heterogeneity between lesions in different labs may be a result of differences in dosage and histological analysis. For the purpose of this thesis we have chosen to use quinolinic acid given the greater degree of specificity associated with this toxin.

An alternative in recent times is the transgenic mouse models of the disease which carry mutated forms of human huntingtin. A transgenic mouse model will have a robust phenotype, may have a rapid disease onset and progression, a well defined quantifiable neurobehavioural abnormality, a neuropathology that mirrors the human disease and limited variability. Mouse models have been generated which have a mutant HD gene or HD gene fragment that has been added to the normal mouse genome, that already has two wild-type mouse HD genes, the transgenic mouse (Hodgson et al., 1999; Mangiarini et al., 1999; Reddy et al., 1998; Schilling et al., 2004) or with an endogenous mouse HD gene engineered to express an abnormally long polyglutamine tract, knock-in animals (White et al., 1997). These mouse models of the disease develop pathological and phenotypic features of HD such as nuclear

and neuronal aggregates. Mice expressing full length mutant huntingtin develop inclusions in the striatum only, whereas mice expressing only exon 1 of the mutant gene developed inclusions in most brain regions (Li et al., 2001) but, generally little or no striatal cell loss. A transgenic rat model of the disease with 51 CAG repeats and which exhibits pathology that closely mirrors that of the human, being slowly progressive and with late onset has been developed (von Horsten et al., 2003). This first rat model of the disease now allows a more detailed long term assessment of the disease progression to be measured. However, as with the transgenic mouse models there is no clear neuronal loss reported in the striatum and so the use of these animals for the assessment of neural transplantation is not ideal.

1.5.2 Transplantation in animal models

Neural transplantation experimentation began as early as 1890 when W. Gilman Thompson first described an attempt to transplant large segments of cortical tissue from dogs into the cortex of dogs (Thompson, 1890). However, the breakthrough in the field came in 1979 when it was first reported that neural transplants of primary foetal tissue could partly restore functionality in animal models of Parkinson's disease (Bjorklund and Stenevi, 1979; Perlow et al., 1979).

1.5.2.1 Primary grafts

Following on from work in PD models, the first success in a HD model was reported in 1981 when it was shown that embryonic rat tissue transplanted to the excitotoxically lesioned rat striatum could survive over prolonged periods of time varying from 4-16 weeks. It was also demonstrated that the grafted tissue was capable of restoring choline acetyltransferase (ChAT) and glutamic acid

decarboxylase (GAD) levels in the lesioned striatum when compared to lesion only controls (Schmidt et al., 1981). Following this, Isacson and Deckel both reported graft induced amelioration of locomotor hyperactivity induced by excitotoxic lesions (Deckel et al., 1983; Isacson et al., 1984). These early studies reported functional improvement in grafted animals on delayed alternation tasks in the T-maze and there was an amelioration of nocturnal hyperactivity that is observed in lesioned animals (Deckel et al., 1986a; Deckel et al., 1986b; Isacson et al., 1986; Deckel et al., 1983). However such studies failed to show an effect of the graft on the induced hyperactivity following amphetamine or apomorphine injection (Deckel et al., 1986b; Deckel et al., 1988).

Primary rat striatal tissue grafts placed in the lesioned striatum have been shown to grow (Isacson et al., 1984; Isacson et al., 1985), and will mature with a time course similar to that seen during normal development of the striatum, thus reaching their final size by 6-8 weeks (Labandeira-Garcia et al., 1991). The success of neural transplantation in this context depends on accurate dissection of the developing striatum (see Figure 1.4), at the appropriate gestational age, and for the preparation to be optimised to maximise cell viability. The greatest concentration of cells with the striatal characteristics of being positive for the dopamine- and adenosine 3':5'-monophosphate regulated phosphoprotein (DARPP-32) (see section 1.4) is derived from the LGE (Deacon et al., 1994), and striatal interneurons are predominantly derived from the MGE (Olsson et al., 1998), but it is still unclear as to whether maximal functional benefits will be achieved by transplanting cells from selective dissection of the LGE (Nakao et al., 1996) or by implantation of cells derived from

both parts combined (the so-called 'whole ganglionic eminence', WGE) (Fricker et al., 1997a; Watts et al., 2000b). We require more empirical studies to decide this tissue.

Figure 1.4

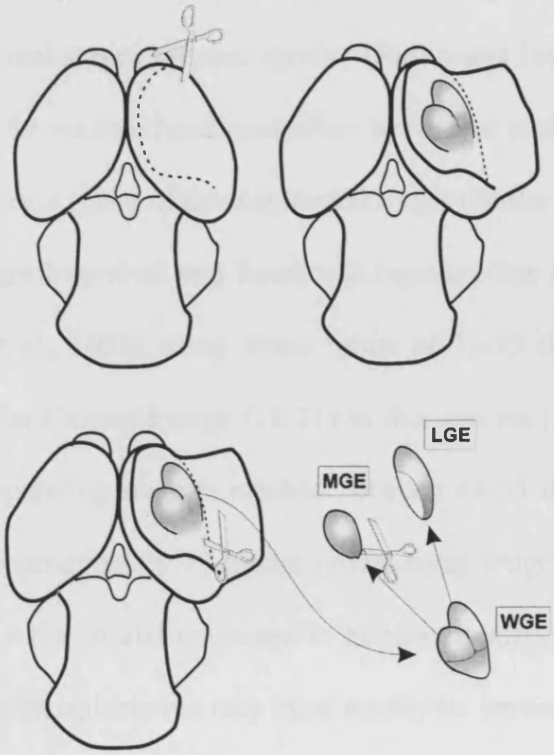


Figure 1.4 Schematic dissection of the whole, medial and lateral ganglionic eminences (WGE, MGE and LGE) from the developing fetal brain (Rosser and Dunnett, 2001).

1.5.2.2 Tissue dissection for transplantation

The gestational age of the tissue is known to be an important factor in determining appropriate neuronal differentiation and optimal survival of the grafts. Fricker et al has shown that grafts derived from younger E14 foetal donors (corresponding to the stage of peak neurogenesis in the developing ganglionic eminence) and transplanted

into adult rat hosts yield larger grafts, better differentiation of the DARPP-32 phenotype, and improved recovery on tests of skilled paw use than do grafts from older foetuses. In the light of these findings, recent pilot clinical trials of cell transplantation in HD in France, the USA and the UK all use tissue of embryos from foetuses at approximately 6-9 weeks post conception (Rosser et al., 2003; Bachoud-Levi et al., 2002a; Hauser et al., 2002b). To date, the staging has been determined by analogy of developmental stages between species (Butler and Juurlink, 1987). Thus, the E14 foetuses used for optimal functional effect by Fricker et al (above) correspond to stage 18 in the Carnegie series of developmental stages (Butler and Juurlink, 1987). A similar pattern of graft survival and functional recovery has been reported in the marmoset (Kendall et al., 1998) using donor tissue of 73-75 days gestation which corresponds to a similar Carnegie stage (18-21) in this species (Annett et al., 1997). In humans, the corresponding stage is reached between 44-53 days post conception (when foetal size is approximately 13-24mm crown-rump length, CRL) (Butler and Juurlink, 1987). Since the striatal eminence in humans is difficult to dissect below 20mm CRL, tissue for transplantation may most readily be harvested towards the end of this time window. Although validated by showing good survival of tissues at this stage of development when xenografted into immunosuppressed rat hosts, the optimal gestational age for human foetal striatal tissue has not yet been systematically determined experimentally. Animal xenotransplantation studies of human to rat have found surviving grafts using tissue derived from human fetal donors as large as CRL 110mm. Although this may suggest that human fetal striatal tissues exhibits significant plasticity over an extended gestational period, older tissues have not yet been shown to produce functional benefit in xenograft experiments. At this early stage of progress of the field, optimisation of tissue preparation procedures,

comparing parameters of survival, differentiation and functional effect, is required to address these ambiguities.

Preparing the tissue as a cell suspension after dissection involves mechanical dissociation of the tissue and Fricker et al (Fricker et al., 1996) found that trypsinisation of rat striatal tissue prior to dissociation resulted in larger surviving grafts that also showed improvements in rotational behaviour. Moreover, these grafts contained more striatal tissue and more DARPP-32 positive medium spiny neurons than grafts implanted as tissue fragments. However, the issue as to whether cell suspension or tissue pieces provide optimum grafts remains ambiguous. There is only one study that examines this issue directly, and it reported no histological differences and a modest improvement in functional recovery on one test in animals receiving tissue fragment grafts compared to suspension grafts prepared from the same rat striatal donor tissue (Watts and Dunnett, 2000; Watts et al., 2000a).

1.5.2.3 Graft Integration

The integration of the grafted tissue has been demonstrated using anterograde and retrograde tracing methods that allow both afferents and efferents from the graft to be examined (Figure 1.5). Afferent connections from the cortex to the graft have been described using anterograde tracers in the frontal cortex and also by administering retrograde tracers to the graft (Wictorin and Bjorklund, 1989). The physiological activity of the cortico-graft connections has also been investigated. Electron microscopy has shown that these afferents do form synapses (Clarke and Dunnett, 1993; Xu et al., 1989) and furthermore, electrical stimulation of the cortex resulted in the expression of the immediate early gene *c-fos* within DARPP-32 positive areas of

the graft although the distribution of Fos protein was uneven when compared to the contralateral non-lesioned striatum (Labandeira-Garcia and Guerra, 1994; Liste et al., 1995). Other afferents to the graft include TH-positive terminals that terminate specifically in P-zones, and synapse with the medium spiny neurons (Clarke et al., 1988; Clarke and Dunnett, 1993; Graybiel et al., 1989a). In accordance with this, amphetamine induced rotation and its induction of *c-fos* indicates that there is an active dopaminergic input to the striatal graft. As well as the dopaminergic input there is also evidence for a serotonergic input from the raphé and thalamic nuclei (Wictorin, 1992).

Using similar methods of anterograde and retrograde tracing the efferent projections from the graft have also been investigated. Efferents have been studied in the primary striatal targets, mainly the globus pallidus and the substantia nigra *pars reticulata.* The efferents to the globus pallidus are from the GABAergic cells of the P-zone (Wictorin et al., 1989b; Wictorin et al., 1989c; Campbell et al., 1995c). It has been shown that, in response to a QA lesion there is an increase in spontaneous electrical activity in the pallidal neurons which is reduced 4 weeks following a striatal graft (Nakao et al., 2000). Likewise a restoration of GABA in the GPe by striatal grafts has also been reported using biochemical techniques (Sirinathsinghji et al., 1988). However, efferents to the substantia nigra are more restricted as this would require the graft cell fibres innervating the host white matter and travelling considerable distances (Nakao and Itakura, 2000). Anterograde transport experiments (Wictorin et al., 1989b; Zhou and Buchwald, 1989) as well as retrograde tracer experiments (McAllister et al., 1989) reported no connectivity between the graft and the nigra and only occasionally (Campbell et al., 1995c; Wictorin et al., 1989c) have retrogradely

injected cells been identified in the graft. However, it is important that one realises that these experiments were carried out in an allograft paradigm where accurate labelling of the grafted cells is difficult due to the inability of reliably identifying graft from host tissue. In comparison, xenografted striatal grafts which can be identified with the use of species specific markers have been shown to send long projections from the graft and as such may be an alternative for circuit reconstruction.

Figure 1.5

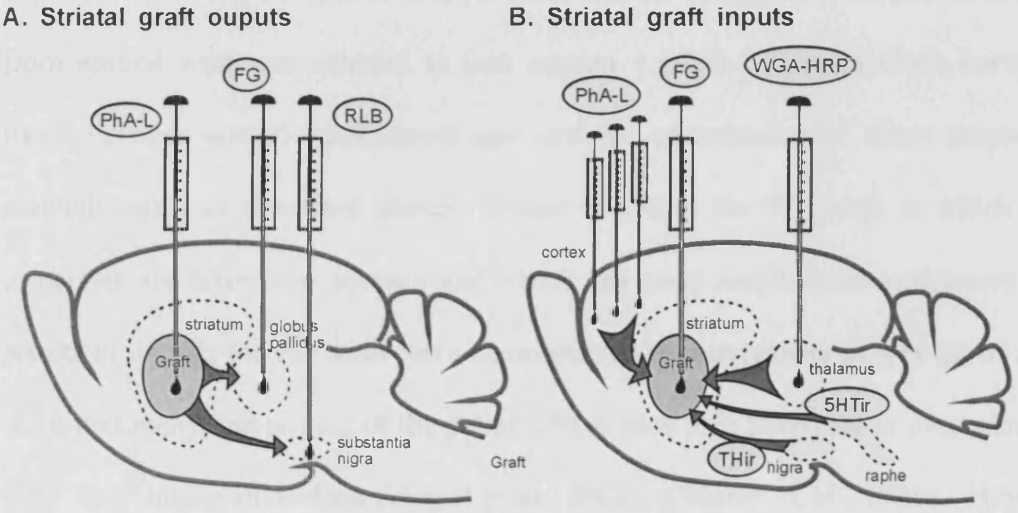


Figure 1.5 Schematic of afferent and efferent connections of striatal grafts using anterograde and retrograde pathway tracing and immunohistochemistry. All major striatal inputs and outputs are established in striatal grafts. Based on Wictorin (1992).

1.6 Clinical Trials

Much of the ground-breaking clinical research on neural transplants was done for PD, beginning in the late 1980s. These trials used primary human foetal mesencephalic tissue as the host tissue and transplanted it into the host striatum, which is the normal target area of these cells. Placing a graft into the substantia nigra is not viable, as the cells are unable to reliably project the distance from the substantia nigra to the striatum. The mesencephalic tissue contains fate-committed dopaminergic neuroblasts which have the capacity to differentiate into fully mature dopaminergic neurones following transplantation, provided that the biological principles determined from animal work are adhered to (see section 1.5.2.1). These include harvesting tissue between specific gestational ages and the optimisation of tissue preparation methodologies as described above. If one considers the PD trials in which these principles are taken into account and which use good longitudinal assessment, then results to date in the PD trials have demonstrated improvements in a range of motor skills and many, but not all, of the patients have been able to reduce or even eliminate their daily intake of L-dopa (Hagell et al., 2002a; Olanow et al., 1996). However, there is variability in the success of this approach, which may be a direct result of variations in transplant methodology as well as differences in patient selection criteria (Freed et al., 2001; Freeman et al., 2000a; Lindvall et al., 1990). Recent trials have also highlighted the possibility of dyskinetic side effects in some patients (Freed et al., 2001), and the reasons for these is currently a topic of active investigation (Hagell et al., 2002b).

Parallel clinical trials of neural transplantation in HD are at a much earlier stage than the PD trials and are currently underway in a small number of centres around the

world. The French trial, based in Créteil, was the first to provide efficacy data based on systematic long-term evaluation of their patients. Three of the five patients, having received bilateral striatal implants, were reported to show substantial improvement over several years (Bachoud-Levi et al., 2002b). More recently there has been an expansion of the French trial to include other French-speaking regions in Europe and a total of 40 patients will eventually receive transplants and will undergo follow-up although no efficacy data is available as yet. In another study in Florida, 6 of 7 patients appeared to show improvement but one declined significantly, so that the overall group changes were not significant (Hauser et al., 2002a). One patient died after 18 months due to cardiovascular disease and post mortem analysis of this patient's brain showed surviving graft tissue that was not affected by the underlying disease progression, at least at this time point (Freeman et al., 2000c). The graft tissue was positive for striatal markers such as acetylcholinesterase, calbindin, calretinin, dopamine and tyrosine hydroxylase. Moreover, there was no sign of immune rejection in the graft region (Freeman et al., 2000b). In the same study 3 patients developed subdural haemorrhages and 2 required surgical drainage (Hauser et al., 2002b). These events may have been related to the stage of disease, which was rather more advanced than for the patients in the French or UK studies, in that more advanced cases of HD tend to have more cerebral atrophy with an accompanying increased risk of intracranial bleeding peri-operatively. Small numbers of patients have received grafts in several other centres with reports of safety (Kopyov et al 1998b, Rosser et al 2002), and although efficacy studies are underway in these centres, systematic reports have not yet been published.

The initial studies of cell transplantation in HD are providing accumulating evidence of the conditions for safety, and preliminary evidence for efficacy. However, the limited availability of foetal tissue and the difficulty in ensuring the high degree of standardisation and quality control when a continuous source of fresh donor tissue is required from elective abortion limits the widespread use of neural transplantation as a practical therapy. Ethical and legislative concerns about abortion and the large number of donors required to support each operation already restrict the number of patients that can receive grafts to a few specialist centres in a restricted number of countries. Moreover, the shifting preference for medical rather than surgical abortions may further limit the availability of tissues to supply even the limited number of programmes already in progress. These issues have stimulated the search for alternative sources of donor cells or tissue that circumvent the problems associated with primary foetal tissue collection.

1.7 Alternative Tissue Sources

A desired characteristic of an alternative cell source is the generation of large stable populations of cells to circumvent the supply issue and also to allow regular characterisation to ensure stability of the quality and character of the tissue, without the need for separate characterisation of each and every collection. Secondly, tissue storage methods need to be refined and validated so that the cells can be delivered on demand to advance optimal clinical management of the recipient, rather than the surgeon and patient being constrained to surgery around an erratic schedule of tissue availability. The trials using primary foetal tissue thus provide a proof of concept of the cell transplantation strategy as the basis for developing a practical therapy using a standardised, quality-controlled source of cells available to any appropriately

equipped neurosurgical facility on demand. Several options are now being investigated as potential sources of donor tissue as described in the next section.

1.7.1 Stem Cells

Stem cells are a potential donor source that has attracted much recent attention. The diverse range of cell types that constitute mature animals arise from a single totipotential stem cell, the zygote. From this totipotential state, germinal populations of proliferative multipotential stem cells at the neurula stage of embryogenesis in the primordia of organs and tissues are established (Larsson). A multipotential cell has the ability to give rise to all the cell phenotypes specific to a particular tissue or organ. Stem cells undergo self-renewal by symmetric division and can also undergo asymmetric division to produce another stem cell and a more differentiated progeny (Morrison et al., 1997; Watt and Hogan, 2000). Some multipotential cells may persist into adulthood, either by remaining quiescent in specific regions of the CNS parenchyma or by continued self renewal (Morrison et al., 1997). Such cells are now referred to as "tissue specific stem cells" (Fuchs and Segre, 2000; Watt and Hogan, 2000). The presence of these cells in the adult may play an important role in maintaining tissue homeostasis by means of a transitory amplifying cell population that is also multipotential and proliferates rapidly in response to signals associated with plasticity, such as following injury (Fuchs and Segre, 2000; Weissman, 2000) (Figure 1.6).

Many definitions of a stem cell take into consideration the attributes described above and also include multipotentialty. However the exact definition remains a matter of dispute and at least some of the ambiguities in the stem cell field result from

differences in usage of the term 'stem cell'. It may therefore be better to use the term 'precursors' rather than stem cells (van der Kooy and Weiss, 2000), which has the advantage of being inclusive, but the disadvantage that it specifies very few necessary or sufficient properties of the cell other than it is not in its final differentiated form. For simplicity, I will continue to use the term 'stem cell' here to refer to a wide variety of proliferating precursors, but I recognise the limitation of this terminology.

Stem cells from a range of sources have potential as donor cells for neural transplantation. However, whatever the source, therapeutic application will require that cells can be directed to differentiate into the precise phenotype required to replace the cells lost to the disease process, and specifically for this thesis, medium spiny neurons for HD. We describe here stem cell sources under consideration as potential donor cells in this context, and the extent to which directed differentiation has been achieved. This list is not exhaustive but covers at least the main categories of stem cells that are currently being explored as alternative cell sources for neural transplantation in HD as well as a number of other neurodegenerative disorders.

Figure 1.6

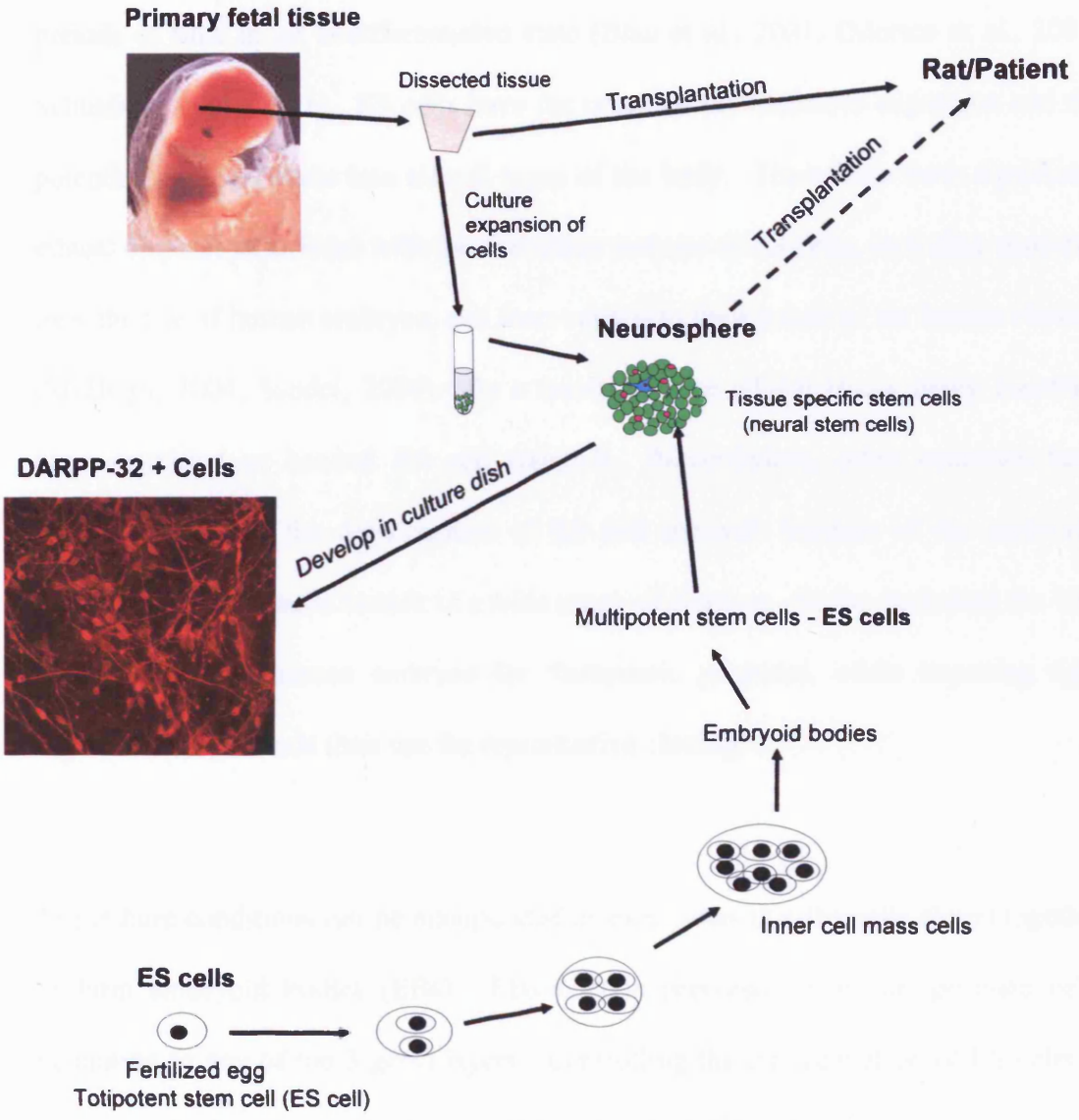


Figure 1.6. Alternative stem cells that may have the potential as a cell source for neural transplantation.

1.7.1.1 Embryonic stem cells

Embryonic stem (ES) cells are isolated from the inner cell mass of the embryo at the blastocyst stage. They are pluripotent and can be propagated in culture for long periods of time in an undifferentiated state (Blau et al., 2001; Odorico et al., 2001; Schuldiner et al., 2001). ES cells have the potential for extensive expansion and the potential to differentiate into all cell types of the body. There have been significant ethical disputes associated with the derivation and use of ES cells, including concerns over the use of human embryos, and fears related to their potential for human cloning (McHugh, 2004; Sandel, 2004). As a result of these ethical issues many countries have restricted or banned ES cell research. Nevertheless, other countries have actively supported the development of ES cell research because of the perceived potential for therapeutic benefit in a wide range of diseases. Some, including the UK, allow cloning of human embryos for therapeutic purposes, while imposing tight regulations to preclude their use for reproductive cloning.

The culture conditions can be manipulated in such a way that the cells clump together to form embryoid bodies (EBs). EBs contain precursors that can generate cells pertaining to any of the 3 germ layers. Controlling the differentiation of ES cells is important, both to derive the target cell populations and to ensure the absence of cells with a continued proliferative potential. There has been progress in directing the differentiation of ES cells down a neuronal lineage, for example by the addition of retinoic acid and nerve growth factor to the medium (Reubinoff et al., 2001; Schuldiner et al., 2001).

A more difficult issue is identifying methods for driving ES-derived neurons down the specific phenotypic lineages required for specific applications. Some progress has been achieved for the dopaminergic differentiation of ES derived neurons with reports of 16-35% tyrosine hydroxylase (TH) positive neurons being generated by the addition of specific factors to the culture medium (Kawasaki et al., 2000; Lee et al., 2000; Okabe et al., 1996; Rolletschek et al., 2001). Expression of the transcription factor Nurr1 enhances the differentiation of ES cells into dopaminergic neurons with reports of 80% TH positive neurons being generated (Chung et al., 2002; Kim et al., 2002; Kim et al., 2003; Grothe et al., 2004; Wagner et al., 1999). However, many of these studies have based their results on the expression of one marker, TH. Whilst this is present in dopaminergic neurons it does not differentiate between the catecholamines, dopamine, noradrenaline and adrenaline and does not indicate that the cells are functional. Detailed analysis of these cells for appropriate receptors as well as dopamine synthesising enzymes storage and uptake molecules (Chung et al., 2002; Kim et al., 2002) needs to be performed to characterise these cells more fully.

Less is known about the ability of ES cells to generate striatal-like cells. Differentiating the cells with chemically defined media containing Iscoves modified Dubelcco's medium and Hams F12 medium in a ratio of 1:1, glutamine, bovine serum albumin, lipids, transferring, insulin and monothioglycerol (Johansson and Wiles, 1995) resulted in a cell population expressing neural fate characteristics typical of the forebrain such a *Dlx5, Dlx1, Lhx5, Tbr1, Pax6, Dbx1, Gsh2, and Gsh1*. In these studies alternative fates were temporally restricted due to a loss of responsiveness to positional cues (Bouhon et al., 2004). In the presence of FGF-2 during the first 8 days

in culture these cells maintain a largely neuronal fate, but with successive passaging an ontogenic drift towards gliogenesis is evident (Kato et al., 2004).

Another important issue is the potential of ES cells to form teratocarcinomas, since remaining undifferentiated ES cells in grafted cell suspension can continue to divide, forming tumours. For example (Bjorklund et al., 2002) grafted a mouse ES cell line into a rat model of PD and reported that 5 out of 25 grafts formed teratoma-like tumours with resulting death of the animals. One method for eliminating undifferentiated cells is by the introduction of suicide genes, such as the E.coli gpt and herpes thymidine kinase (HSVtk), into the cells prior to transplantation. The plasmid vector also contained a neomycin resistance gene that allowed selection of the undifferentiated ES cells as differentiated cells in the presence of the neomycin resistance gene will be resistant to the effects of ganciclovir. Undifferentiated HSVtk positive cells that continue to proliferate can then be destroyed by the conversion of the prodrug nucleoside ganciclovir to its phosphorylated form which is then incorporated into the DNA of replicating cells resulting in apoptosis of the cells (Schuldiner et al., 2003; Fareed and Moolten, 2002). The functionality and efficacy of the differentiated cells will also have to be addressed as well as the possibility of rejection before they can be considered for clinical trials.

1.7.1.2 Embryonic Germ Cells

Embryonic germ (EG) cells are diploid primordial cells that migrate from the posterior endoderm of the yolk sac via the gut mesentery during development thus populating the developing gonads (Molyneaux et al., 2001). Once in the gonads these cells proliferate and finally undergo meiosis to yield spermatozoa or ova. The

population of EG cells taken for culture are obtained during the first trimester from the premeiotic foetal gonads (Shamblott et al., 1998).

Mouse EG cell lines have been found to vary in their properties depending on the day on which they were derived and this may be a result of imprinting which is the inactivation of either the maternal or paternal alleles at a particular locus. Imprinting of genes takes place during migration of the cells to the gonadal ridge, and this appears to have effects on the differentiation of these cell lines *in vivo* and *in vitro* (Durcova-Hills et al., 2001; Durcova-Hills et al., 2003; McLaren, 2001). The mouse-derived EG cells can proliferate for prolonged periods of time in culture in the presence of specific growth factors and are pluripotent and chromosomally stable, thus resembling ES cells.

Less is known about human derived EG cell lines, but early reports suggest that they are not as easy to maintain in culture as mouse EG cells and may spontaneously differentiate *in vitro* (Turnpenny et al., 2003). Little is known of the potential of these cells in relation to neural transplantation.

1.7.2 Tissue-specific neural stem cells

1.7.2.1 Fetal embryonic neural precursor cells (ENPs)

ES and EG cells are a totipotential or multipotential source of cells, and thus require manipulation *in vitro* to direct them firstly to a neuronal fate and furthermore to a striatal-specific phenotype. An alternative approach is to seek to identify stem cells that are already committed to a neural lineage (i.e. tissue-specific), and furthermore,

from an even more restricted lineage - striatal precursors from which it may be easier to drive an explicitly striatal phenotype.

All cells of the adult CNS arise from the neuroepithelium, a germinal layer that surrounds the ventricle of the embryonic brain (Larsen, 1998). *In vivo* fate mapping experiments have been undertaken to demonstrate that multipotential cells play a role in generating the phenotypic diversity of the mammalian CNS and thus multipotentiality is described as the ability of a precursor cell to give rise to all cell types of the nervous system, neurons, astrocytes and oligodendrocytes. By injecting a low concentration of a replication-deficient retroviruses carrying a reporter gene into the forebrain ventricle of the embryo it is possible to infect individual cells and then to use this to determine multipotentiality by assessing the progeny of these cells *in situ* (Luskin et al., 1988). Based on this technique it has been postulated that by E12-E14 most precursor cells in the mouse cortical germinal zone are fate-restricted and generate only neurons or glia (Luskin et al., 1988; Grove et al., 1993). However, it has also been shown by others that the label in some cases may be inherited by both neuronal and glial progeny which would suggest a common precursor cell (Price and Thurlow, 1988).

Detailed study of the multipotentiality of precursor cells in the mammalian CNS is difficult given retroviral labelling can be used, although there are technical constraints; which includes the possibility that two neighbouring unipotent cells may be infected with the label thus leading to misleading interpretation of the clonality of the progeny (Walsh and Cepko, 1993; Walsh and Cepko, 1992). *In vitro* analysis of precursor cells using retrovirus labelling has helped to circumvent some of these

problems and have confirmed the presence of multipotential cells in the developing mouse cortex (Williams and Price, 1995; Williams et al., 1991). Another method is to culture individual cells and follow their progeny and such experiments have shown for example that murine cortical cells differentiate into neurons and glia and also have the potential to self renew (Temple, 1989; Davis and Temple, 1994; Temple and Davis, 1994). These experiments also highlight the importance of epigenetic factors for differentiation and epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) are strongly implicated in this regard.

Reynolds and Weiss have shown that E14 mouse striatal cells grown in the presence of EGF result in the proliferation of free floating spheres "neurospheres" (Reynolds and Weiss, 1992b) (Figure 1.7). These cells were able to differentiate into neurons and glia and were also shown to express the maker nestin which is an intermediate filament characteristic of neuroepithelium. To ensure that the differentiated progeny observed in these experiments were not from a separate population of lineage restricted progenitor cells it was subsequently shown that dissociated neurospheres cultured as single cells with EGF were able to form new multipotential spheres (Reynolds and Weiss, 1996).

Clonal analysis in the presence of FGF-2 has shown it to be mitogenic for ENPs (Drago et al., 1991; Gensburger et al., 1987; Ray et al., 1993; Ray and Gage, 1994; Richards et al., 1992; Vicario-Abejon et al., 1995). These studies have shown that ENPs will self renew and differentiate into neurons and glia in the presence of FGF-2.

As well as stimulating cell proliferation in culture, FGF-2 and EGF act sequentially on the regulation of differentiation (Vescovi et al., 1993). FGF-2 is known to enhance the neuronal differentiation of the cells whilst EGF promotes astroglial differentiation (Armstrong et al., 2000). Several other growth factors have the potential to enhance the neuronal differentiation of these cells down particular lineages, including nerve growth factor (NGF), insulin-like growth factor (IGF) and tumour necrosis factor (TNFα) (Arsenijevic et al., 2001; Cattaneo and McKay, 1990; Santa-Olla and Covarrubias, 1995; Tropepe et al., 1997). Identifying an appropriate growth factor cocktail appropriate to the phenotype associated with each particular application may be a necessary prelude to using these cells for transplantation.

Figure 1.7

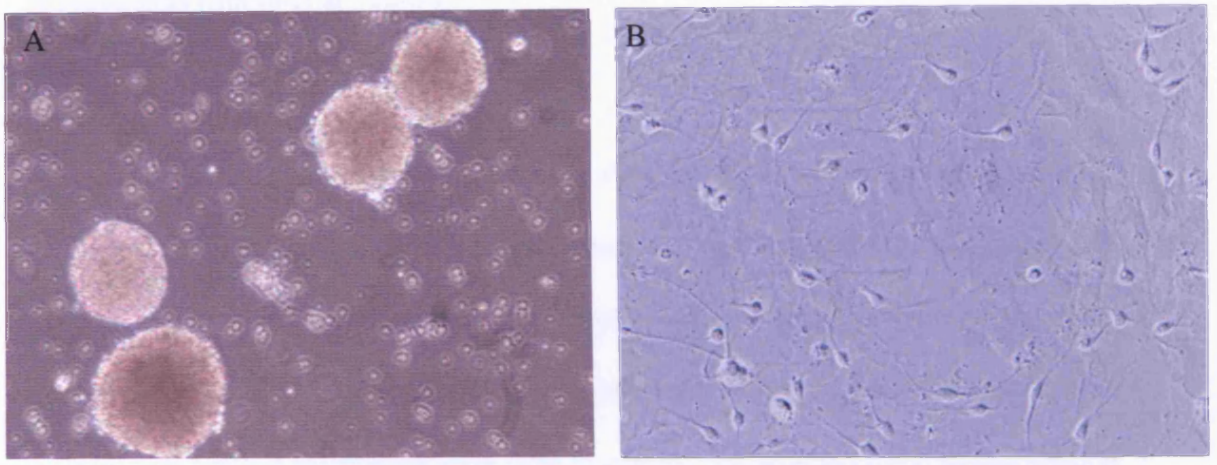


Figure 1.7 A, Fetal ENPs can expand in culture to form free-floating spheres of cells ("neurospheres"), each of which contains several thousands of cells. B, Upon mitogen withdrawal these cells can differentiate into neurons, astrocytes and oligodendrocytes.

ENPs have been isolated from several species including mouse (Kilpatrick and Bartlett, 1993; Murphy et al., 1990; Reynolds and Weiss, 1992a), rat (Smith et al., 2003; Svendsen et al., 1995; Svendsen et al., 1997b), pig (Armstrong et al., 2001b; Armstrong et al., 2003b; Armstrong et al., 2002; Jacoby et al., 1997; Talbot et al., 2002), and human (Armstrong et al., 2000; Burnstein et al., 2004; Carpenter et al., 1999; Carpenter et al., 2003; Englund et al., 2002b). There is cross species variation in the proliferative potential of ENPs (Svendsen et al., 1997b): Rat cells entered a state of senescence after a relatively short period of time, 30-40 days, in contrast to mouse and human cells that have the potential to proliferate for much longer periods of time in culture (Svendsen et al., 1997b). The underlying reasons for these

differences are unclear, but may be related to species differences in tissue culture requirements (Smith et al., 2003).

Molecular characterisation of foetal ENPs *in vitro* has shown that they retain a degree of their site-specific identity when environmental cues are absent but when co-cultured with cells of different origin they can adopt a new fate (Fricker et al., 1999; Parmar et al., 2002). Expression of genes associated with striatal development such as IsletI and Er81 is maintained over time in culture, but with neuronal differentiation expression of striatal specific neuronal markers such as DARPP-32 and Islet1 are lost, although they do express homeobox transcription factors Dlx and MEIS2, which are associated with ventral forebrain development (Parmar et al., 2002; Skogh et al., 2003). Thus, it appears that expansion of ENPs in culture may restrict the differentiation potential of the cells. Further evidence for this comes from transplantation of ENPs into disease models which demonstrate that after a short period of expansion ENPs can survive post-transplantation; but that this is compromised by longer expansion times (see section 1.7.3.1.1). One interpretation of these findings is that positional information is lost with continued expansion so that when long-term expanded cells are placed in an environment such as the adult CNS, they are not exposed to the developmental signals that they would see in the developing brain and are thus unable to differentiate into neurons appropriate to the site from which they were derived (for example medium spiny neurons from striatally-derived ENPs). However, when grafted to the neonatal brain, similar cells appear to respond to developmental signals and regional determinants by differentiating in a site-specific manner (Englund et al., 2002a; Englund et al., 2002b;

Rosser et al., 2000) suggesting that they retain the capacity to respond to developmental signals if they are present.

1.7.2.1.1 Transplantation of expanded fetal ENPs

Grafts of ENPs have not been as extensively studied as primary foetal grafts but the literature suggests that the time that these cells are expanded for in culture may have an important impact on the survival of the cells *in vivo*. Zietlow (Zietlow et al., 2005) has shown that cells expanded for 4 weeks (short term expanded, STE) compared to those expanded for 20 weeks (long term expanded, LTE) will result in surviving grafts after 20 weeks *in vivo*. Graft disappearance was reported at 4 weeks *in vivo* and this was higher in the LTE group (25%) than the STE group (14%) thus suggesting that the mechanism leading to LTE graft death is already effetive at 4 weeks *in vivo*.

Svendsen and colleagues grafted 13 week human foetal tissue that had been expanded in culture in the presence of EGF for 10 days (unpassaged) or for 28 days (Svendsen et al., 1996). Grafts were placed into the 6-OHDA lesioned brain and 4 weeks post grafting it was found that 10 day expanded cells survived and formed large graft masses whereas those expanded for 28 days failed to form a discernable graft mass. In a subsequent study the same group reported that human foetal tissue expanded for 14-28 days in the presence of EGF and bFGF gave surviving grafts after 20 weeks *in vivo* (Svendsen et al., 1997a). Most of the cells in such grafts were found to contain astrocytes that had migrated out from the graft core and there were only few neurons reported.

McBride and colleagues have reported striatal grafts of human cortical tissue that was expanded in culture for 12 weeks and maintained in vivo for 8 weeks. In these grafts

surviving human nuclear antigen (HuNu) stained cells were observed in most grafts and were seen to be widely distributed throughout the striatum. Migration of the grafted cells was also reported with HuNu positive cells observed in the globus pallidus, substantia nigra pars reticulata and the entopeduncular nucleus, however on no occasion were there HuNu positive cells reported on the contralateral side of the brain (McBride et al., 2004). Grafts of human foetal striatal tissue expanded in culture for 10-12 days to the lesioned striatum for 12 weeks sent out long projections diffusely in the brain (Armstrong et al., 2000) and differentiated into mature striatal neurons based on their expression of striatal markers such as DARPP-32.

The migration potential of ENPs post-transplantation has also been studied and it has been found that they have the potential to migrate out from the graft core. Fricker and colleagues have shown that human ENPs when placed in neurogenic regions of the adult brain will migrate to the appropriate targets of that region and differentiate into region specific neurons, as well as this, the same study also reported non-specific migration of the glial cells to the non-neurogenic striatum (Fricker et al., 1999).

From these studies and those in table 1 it is clear that ENPs have the potential for neural transplantation. However, for this to become successful it is imperative that we first optimise the conditions in which these cells are expanded so as to increase the frequency at which these cells differentiate into the appropriate phenotype. One possibility that is now being investigated is pre-differentiation of the cells prior to transplantation (Yang et al., 2004; Burnstein et al., 2004; Le Belle et al., 2004; Studer et al., 1998). Burnstein and Le Belle have both reported increased neuronal survival and migration in grafts of pre-differentiated cells. Studer and colleagues reported

intrastriatal grafts of differentiated dopamine cells can induce functional recovery in a Parkinson's disease model. However Yang and colleagues transplanted human neural progenitor cells to the 6-OHDA lesioned striatum and were unable to report TH-positive neurons in their grafts and so it is evident that much work is needed to establish the potential of this method for transplantation.

Studies of non-genetically modified ENPs transplanted into the CNS

Growth factor responsive neural progenitors					
	Study	*Cells*	*Age and status of host*	*Notes*	*Results*
Adult unlesioned Host	Sabaté et al., 1995	Human (bFGF)	Adult intact	Cells propagated for 11days in vitro and maintained in vivo for 2-3 weeks	Surviving grafts only when $6x10^5$ cells grafted
	Winkler et al., 1995	Murine (EGF)	Adult intact	Cells proliferate and migrate in response to EGF infusion	Predominantly non-reactive glia
	Fricker et al., 1999	Human (EGF,bFGF,LIF)	Adult intact	Extension of axons to GP and toward SN	Site-specific neuronal and glial differentiation
	Fricker-Gates et al., 2000	Murine (EGF)	Adult intact	Cells proliferate and migrate in response to EGF infusion	Predominantly non-reactive glia
	Messina et al., 2003	Human (bFGF)	Adult intact	Cortical cells were grafted with subpopulation of nestin expressing cells and compared to nestin positive only grafts	More cell division with more neurons and astrocytes in the mixed graft paradigm
	Jain et al., 2003	Human (EGF,bFGF,LIF)	Adult intact	Cells propagated for 6-10weeks in culture	Number of neurons in vivo decreased with time in vitro but no difference in migration. More neurons migrated from hippocampus
	Le Belle et al., 2004	Human (EGF,bFGF,LIF)	Adult intact	Transplanted both differentiated and proliferating cells. Differentiated cells were pre-treated to boost glial energy stores and increase neuronal survival	Transplanted pre-differentiated cells were seen to respond to local cues in the hippocampus but showed less migration

	Study	Cells	Age and status of host	Notes	Results
Lesioned brain	Svendsen et al., 1996	Rat (EGF)	Adult IA lesioned	Only a little migration toward lesion, Cells were propagated for 10-23 days in vitro	No evidence of neuronal differentiation, mainly glial response
		Human (EGF)	Adult nigrostriatal lesion		TH-positive cells in graft
	Svensen et al., 1997	Human (EGF,bFGF)	Adult nigrostriatal lesion	Propagated for only 14 days in vitro	TH-positive neurons in 2 animals that showed reduction in rotation
	Studer et al., 1998	Rat (bFGF)	Adult nigrostriatal lesion	Propagated in vitro for 6-8 days and pre-differenitated for 7 days	Significant functional improvement and TH+ cells
	Corti et al., 1999	Human (bFGF)	Adult nigrostriatal lesion	Regulatable expression of the TH transgene	Adenovirus-mediated transfection pre graft
	Vescovi et al., 1999	Human (EGF, bFGF)	Adult nigrostriatal lesion	Differentiated for 6 days in vitro pre-transplantation	Neuroanal differentiation in graft. TH+ cells not reported
	Ostenfeld et al., 2000	Human (EGF, bFGF)	Adult nigrostriatal lesion	Cells propagated for 8weeks in vitro and maintained in vivo for 2, 6, and 20weeks	Dividing cells declined with time post-grafting. No functional effect and NF70 fibres only in low density grafts
	Armstrong et al., 2002	Porcine (EGF,bFGF)	Adult nigrostriatal lesion	Cells transplanted to substantia nigra and striatum and maintained for up to 20 weeks	Dividing cells declined with time post-grafting. No functional effect and NF70 fibres only in low density
	Armstrong et al., 2000	Human (EGF, bFGF)	Adult QA lesioned	Propagated for only 10 days in vitro, diffuse projections not target directed	Site-specific neuronal and glial differentiation
	Burnstein et al., 2004	Human (EGF,bFGF,LIF)	Adult nigrostriatal lesion	Cells propagated for 10weeks in vitro and maintained in vivo for 12weeks	No behavioural effect. Differentiated and undifferentiated cells. More neurons in differentiated grafts
	McBride et al., 2004	Human (EGF,bFGF)	QA lesion	Cells porpagated for 12weeks in vitro and maintained in vivo for 8 weeks. One group received CNTF prior to transplantation	Neurons showing migration to GP, EPN and SN. Improvement on cylinder test.
	Zietlow et al., 2004	Human (EGF, bFGF) Murine (EGF, bFGF)	IA lesion or Adult intact	Cells propagated for 4 or 20 weeks in vitro and in vivo for 4 or 20 weeks	Long term expanded cells did not survive 20 weeks post transplantation.

	Study	Cells	Age and status of host	Notes	Results
Embryonic brain	Brustle et al., 1998	Human (EGF,bFGF)	Embryo intact	Propagated for up to 7 weeks in vitro and maintained for up to 7 weeks in vivo	Neurons with regional morphology, astrocytes and oligodendrocytes
	Winkler et al., 1998	Murine (EGF)	Embryo intact	Cells placed in lateral ventricle, extensive migration throughout brain	Predominantly non-reactive glia
Neonate Brain	Flax et al., 1998	Human (bFGF)	Neonate intact	Maintained in vivo for 24hrs-5weeks	Glial differentiation in cortex. Neurons in olfactory bulb and cerebellum
	Rosser et al., 2000	Human (EGF,bFGF)	Neonate intact	Propagated in vitro for 22 weeks and maintained in vivo for 4 weeks	Neurons with regional morphology, better survival in hippocampus than striatum
	Englund et al., 2002	Human (EGF,bFGF,LIF)	Neonate intact	Extension of axons to GP and toward SN	Site-specific neuronal and glial differentiation
	Eriksson et al., 2003	Murine (EGF, bFGF)	Neonate intact	Cells propagated for 5-6 passages and showed mature neuronal morphologies *in vivo*	Site-specific neuronal and glial differentiation
	Parmar 2003	Human (EGF, 10% serum)	Neonate intact	Cells propogated for 11-13 passages and both diferentiated and non differentiated cells were transplanted	Transplanted cells behaved similar to Olfactory bulb cells and expressed such markers
Other	Clarke et al., 2000	Adult Murine (EGF)	Chick embryo and Mouse morula	Propagated for ~14 days in vitro. No haematopoietic cells reported	Better incorporation in chick with donor cells in all three germ layers, immature markers only.

1.7.2.1.2 Adult neural stem cells

Adult neural stem cells (ANSCs) are also a tissue-specific stem cell and are derived from the mature brain. Altman and colleagues provided the first clear evidence, using ^{3}H-thymidine autoradiography, that a low level of neurogenesis is ongoing in the dentate gyrus of adult rats (Altman and Das, 1965). ANSCs have since been confirmed in two main regions of the CNS: the sub granular layers of the dentate gyrus, from where the newly-formed neurons repopulate the dentate gyrus (Gage et al., 1995); and the subventricular zone (SVZ) of the lateral ventricles (Alvarez-Buylla et al., 2002), from where the newly formed neurons migrate via the rostral migratory stream to the olfactory bulb (Lois and Alvarez-Buylla, 1994) (Figure 1.8). More recently it has been reported that neural stem cells may also reside in other regions of the brain, albeit at an even lower concentration, including cortex (Gould et al., 1999; Rietze et al., 2000) and the medial-rostral part of the substantia nigra pars compacta in the lining of the cerebroventricular system of the midbrain (Zhao et al., 2003), although these reports remain controversial (Frielingsdorf et al., 2004).

Figure 1.8

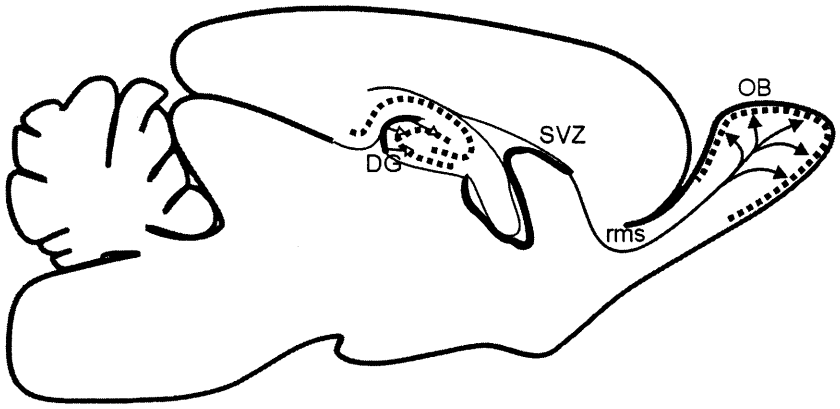


Figure 1.8 Adult neural stem cells have been identified in the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus. The SVZ cells migrate along the rostral migratory stream to the olfactory bulb, whereas newly formed neurons in the DG take up residence within in the granule cell layer. (Dunnett, 2001).

The attraction of ANSCs as a donor supply for neural transplantation would be the possibility of autologous transplants, thus bypassing the immunological issues of graft rejection which can be severe in the case of xenografts and not entirely benign even for allografts. Furthermore, it may eventually be possible to recruit such cells for endogenous repair without a requirement for their isolation and re-implantation. That is, it might be possible to stimulate the resident population of ANSCs to migrate to the site of degeneration, although adult stem cells remain difficult to isolate and grow in culture and the factors that would be required to enhance the proliferation of these cells and their differentiation into the particular phenotypes relevant to the site of degeneration remains unknown.

1.7.3 Trans-differentiation of other tissue-specific stem cell populations

Another approach is to attain trans-differentiation of non-neural tissue-specific stem cell population, the classic one being bone-marrow-derived stem cells. This population have the advantage of being more easily harvested than either foetal or adult neural stem cells, but the disadvantage that they do not by default produce neurally differentiated cells.

During development, mesenchymal stem cells (MSCs) give rise to all mesodermal cell types of the body, including osteoblasts, chondrocytes, adipocytes and muscle cells, and cells with similar characteristics are found in adult bone marrow along with more lineage-restricted cells that contribute to differentiated haematopoietic cells. There is currently controversy as to the precise categorisation of these marrow components (Ratajczak et al., 2004).

There is some evidence that trans-differentiation can be achieved, although this remains an area of dispute. MSCs have been reported to trans-differentiate to ectodermal and endodermal cell fates (Zhao et al., 2002) and, *in vitro,* MSCs have differentiated to form neurons and astrocytes. MSCs transplanted into the rat brain survive and express markers of neuroectodermal cells as well as having a functional effect (Zhao et al., 2002). MSCs are not the only cells able to trans-differentiate, as neural stem cells have also been shown to have this ability, where they were seen to differentiate into muscle (Galli et al., 2000). However, recent evidence suggests that this plasticity may be a result of cell fusion based on studies that have looked at the

potential of MSCs to differentiate into hepatocytes (Wang et al., 2003; Vassilopoulos et al., 2003). This issue will need to be clarified for these cells to be serious contenders for neural transplantation.

Hematopoietic stem cells (HSCs), also derived from the bone marrow, continually reconstitute the blood and are the best characterised of the tissue-specific stem cells. Two classes of HSC have been identified in mouse, those that survive for around 2 months, (the short term, ST-HSC), and those that survive for greater than 6 months, (the long term, LT-HSC) (Blau et al., 2001). Fluorescence-activated cell sorting (FACS) has been used to positively select cells based on the expression of specific cell surface markers. HSCs can be highly enriched up to 10,000 fold and then transplanted into the bone marrow of patients (Lagasse et al., 2001) for the treatment of oncogenic blood diseases. In an animal model of spinal cord injury, HSCs have been shown to survive for 5 weeks after transplantation, differentiate into astrocytes, oligodendrocytes and neuronal precursors and show improvement in functional behaviour using hindlimb motor function (Koshizuka et al., 2004), although no mature neurons were identified.

Human umbilical cord blood is easily retrieved following labour without the risk of harm to the mother or child and has been reported to contain multipotential progenitor cells that apparently have the ability to trans-differentiate into neuronal and glial cells (Sanchez-Ramos et al., 2001). Transplantation of these cells into the neonatal and adult brain have shown potential to survive and differentiate into neurons and glia (Li et al., 2004; Willing et al., 2003; Zigova et al., 2002; Nan et al., 2005; Sanberg et al., 2005). It may be that intravenous delivery rather than neural transplantation will be a

more advantageous method of administering these cells for therapeutic benefit, based on a study by Willing et al (2003) where there was significant improvement in certain behavioural tasks when compared to animals receiving neural transplants of cells directly to the striatum. However, further studies are necessary to validate the potential of these cells and again, the issue of cell fusion needs to be addressed in this context.

1.7.4.Xenogenic tissue

Xenotransplantation offers the opportunity of breeding animals for foetal striatal tissue donation under conditions where the supply can be regulated according to demand; where the breeding stock is inbred, well characterised and controlled for pathogens; and where tissue collection and preparation can be undertaken under standardised sterile GMP conditions. The most likely donor candidate is porcine tissue, the advantages being: the extensive experience of animal husbandry within this farm species; the reliability of breeding; the large size of the litters; the possibility of sterile collection under standardised conditions; the comparable size and time course of development of the pig and human brain; and the potential application of transgenic technology to porcine tissue, which would open up the possibility of genetic manipulation, for example to modify the immunogenicity of transplanted tissue.

Transplantation of xenogeneic tissues into the immunosuppressed host CNS has been performed using a number of species, for example human to rat, pig to rat, rat to mouse and vice versa (Armstrong et al., 2002; Deacon et al., 1999; Galpern et al., 1996; Garcia et al., 1995; Isacson et al., 2001; Svendsen et al., 1997b). Both primary

and expanded tissue graft experiments have been reported using xenogenic tissue. The grafted tissue has been found to survive transplantation, axonal and glial fibre projections from the grafts, and make synapses with the host brain.

Studies of the potential of porcine tissue to achieve brain repair have been carried out predominantly in PD models. Primary tissue grafts have been shown to integrate into the host environment but even with Cyclosporine A (CsA), a slow rejection process ensues, and finding ways to overcome the issue of graft rejection is the subject of much investigation. Primary porcine VM tissue grafted into cortically lesioned neonate brains survived, integrated with the host brain, and sent out long axonal projections, thus showing the potential for porcine tissue to respond to rat axonal guidance factors (Castro et al., 2003). Similar findings have been reported following placing porcine VM tissue into the lesioned cortex of adult rats (Garcia et al., 1995) and into the striatum in animal models of HD and PD (Isacson et al., 1995b). There has been good functional improvement with grafts placed in the striatum of a 6-OHDA lesioned rat model based on compensation on the amphetamine rotation test (Larsson et al., 2000a). In these studies, CsA was used as an immunosuppressant to protect the grafted tissues from host rejection. Further improvement of graft survival was achieved by the addition of caspase inhibitors, which increased the number of TH positive cells in the grafts by 2.5 fold (Cicchetti et al., 2002). Again, donor age is an important issue. Comparison of donor ventral mesencephalic tissue ranging in ages from E24 to E35, indicated that E26-E27 embryos gave the highest yield based on TH differentiation of the tissue (Barker et al., 1999; Larsson et al., 2001a; Molenaar et al., 1997).

Clinical studies of CNS xenotransplantation are limited. Primary porcine embryonic striatal tissue has been transplanted into the caudate and putamen of 12 immunosuppressed PD patients with some clinical improvements reported, although there was little convincing evidence of graft survival (Isacson et al., 2001). The immune response from these grafts was more vigorous than that seen in human to rodent models. One patient died 7 months post-operatively for reasons unrelated to the graft, and was found to have very small numbers of surviving neurons in the graft region, raising the possibility that the majority had been rejected. In the same series, 12 HD patients received porcine striatal grafts but, again, there was little evidence of graft survival or functional effect. Twelve months of post-operative analysis of these patients demonstrated no change in the mean total functional capacity score (Fink et al., 2000a).

Striatal allografts of primary foetal tissue in the HD lesion model have been shown to send out projections in the host brain as evidenced by anterograde and retrograde tracing methods (Wictorin et al., 1989b; Wictorin, 1992) (see Figure 1.6). Similarly xenotransplantation of striatal tissue has also been shown to send out projections in the host brain (Olsson et al., 1997; Wictorin et al., 1991; Armstrong et al., 2001b; Armstrong et al., 2000). Neurofilament staining using species-specific antibodies for xeotransplanted tissue has been successful in revealing the extent and richness of short and long distance outgrowth from striatal grafts (Armstrong et al., 2000; Wictorin et al., 1991). However, it is difficult to assess whether or not this is different to the allograft situation, as there are many fewer tools for discerning allograft projections from host projections and thus allowing a comparison to be made.

Two key issues need to be resolved for xenografts to progress as to practical therapeutic trials. The first relates to the fact, as illustrated by the first pilot clinical trial reported above, that xenografted tissue is largely rejected in the absence of effective immune protection. Two alternative strategies were adopted in the Diacrin trial – daily treatment with CsA or treatment with an antibody against major histocompatability complex 1 (MHC 1) to block the host T cell response (Fink et al., 2000b). There is no clear evidence that either strategy proved effective for yielding good cell survival in patients, and it is surprising that the study had progressed on the basis that preliminary reports of the same strategies in primates were equally ineffective. Combination immunoprotection strategies to promote xenograft survival are an area of active research (Harrower et al., 2004b; Armstrong et al., 2001a) but a clear optimal protocol that will allow reliable long-term survival of xenografted neural tissue in the adult brain in the majority of subjects has not yet been defined.

The second key issue that requires resolution relates to safety of xenografted tissues. In the light of the recent spread of bovine spongiform encephalopathy to man in the form of new variant Creutzfeld-Jacob disease, and the difficulty in controlling the spread of animal pathogens, as exemplified by the recent UK foot-and-mouth epidemic, there is widespread concern world-wide about the difficulties of eliminating the possibility of transmitting animal diseases to man. This may be particularly risky in the context of transplantation of tissues directly into the immunosuppressed CNS. The concern is not just for the recipient but, in the case of porcine endogenous retrovirus (PERVs), whether direct transfer into the brain might provide a route of transmission that allows virus mutation into new forms of viruses that give rise to unpredicted new diseases in man, even giving rise to *de novo* epidemics. Although

the chances of such mutation are recognised to be very low, the cost of occurrence could be devastatingly high. Moreover, the risk of generating a new disease by an unknown mechanism is one that it is impossible to absolutely exclude by any known safety screen. The regulatory climate is consequently such that any novel xenograft approach is unlikely to gain approval for trial in the foreseeable future, at least in Europe. In the absence of having suffered the same major BSE, CJD and FMD epidemics, US regulations, although strict, are somewhat more permissive, with the result that most academic and commercial research of developing xenotransplantation as a therapeutic strategy for the CNS has moved westwards across the Atlantic over the last 5 years.

The capacity for embryonic porcine neural tissue to be expanded in culture is dependant on gestational age of the tissue, with E22 tissue expanding and surviving transplantation more robustly than that of tissue from an E27 embryo (Armstrong et al., 2003a). Following transplantation, expanded neural porcine tissue differentiated into neurons and sent out projections to the appropriate target areas. Moreover, expanded porcine cells survived for longer periods of time in the non-immunosuppressed rat host than did primary porcine CNS tissue (Armstrong et al., 2001b). Thus, it may be that expansion of the tissue confers a benefit in terms of reduced immunogenicity, and there is some supporting *in vitro* evidence that the expression of surface histocompatibility antigens is reduced in porcine neural stem cells (Harrower et al., 2004a). However, in common with other stem cell sources (see below), there remains a significant problem in the expanded cells retaining a specific striatal or dopaminergic phenotype limiting their functional effects until this can be overcome.

1.7.5 Genetically Engineered Cells

A variety of cells may be engineered *in vitro* either for the purpose of producing molecules of potential importance for CNS release (for example, in the form of polymer encapsulated cells, as below), or to alter the properties of a cell to render it potentially useful for circuit reconstruction. Of course, these strategies are not necessarily mutually exclusive - trophic factor support may be crucial for transplanted cells to survive and integrate in the host brain, and genetically engineering cells to release trophic factors in the graft region is one potential method for optimising graft survival.

The herpes simplex viral vector was the first virus to be tested as a method of introducing genes into the adult CNS (During et al., 1994; Song et al., 1997; Fraefel et al., 1996). More recently, other viral vectors have been introduced, including adenovirus, the recombinant adeno-associated virus (rAAV), lentivirus and pseudotyped vectors. The rAAV vector is more efficient than the HSV in that it is possible to achieve much higher levels of expression. The use of such vectors has allowed genes to be transferred to a specific group of cells in the CNS (Janson et al., 2001), and has provided support for the efficacy of factors such as GDNF for PD (Eslamboli et al., 2003; Kirik et al., 2000; Mandel et al., 1999; Mandel et al., 1997) and CNTF for HD (Regulier et al., 2002; Kahn et al., 1996; Emerich and Winn, 2004; Mittoux et al., 2002).

Polymer capsules have been considered as a system for trophic factor delivery to the CNS as they have the advantages of being relatively cheap to produce and can also be removed from the CNS as required, but the major drawback is that the effect is not long lasting (Emerich et al., 1994). Where a limited amount of a protein is required for relatively short periods of time, polymer microspheres are an attractive alternative as they are biodegradable and subsequent surgical procedures are not required for retrieval (Date et al., 2001). However, improvements in the duration of release have been obtained by the use of encapsulated cells engineered to produce the desired molecules (Emerich et al., 1997a; Emerich, 1999). Here, cells engineered to secrete specific substances such as neurotrophic factors are protected from the host immune system by a semi-permeable selective biocompatible outer membrane (Emerich et al., 1994; Emerich et al., 1996; Emerich et al., 1997b; Emerich et al., 1998; Emerich and Winn, 2004). The outer membrane allows the entry of nutrients to the cells whilst also allowing the exit of neuroactive molecules. The advantage of this strategy is that it allows for the implantation of xenogeneic cells, which may be much easier to obtain or engineer than human cells. This approach has been used for delivery of factors such as GDNF in animal models of PD (Date et al., 2001; Sautter et al., 1998) and CNTF in animal models of HD (Emerich et al., 1997a).

In the case of HD there have been several studies using polymer encapsulated cells for the delivery of CNTF. Baby hamster fibroblasts have been genetically modified to produce hCNTF and incorporated into polymer capsules (Anderson et al., 1996; Emerich et al., 1996). Both rodent and primate studies have been carried out incorporating this method (Anderson et al., 1996; Emerich et al., 1996; Emerich et al., 1997b; Emerich and Winn, 2004; Kordower et al., 2000; Mittoux et al., 2000). These

animal studies suggested that CNTF can protect striatal neurons against subsequent damage from an excitotoxic lesion. As well as protecting specific populations of striatal neurons from lesion-induced cell death, behavioural improvement was observed on skilled motor and cognitive tasks when compared to control animals. Encapsulated CNTF released by BHK cells is now in clinical trials (Bachoud-Levi et al., 2000). Nevertheless, the use of encapsulated cells for the delivery of growth factors and neurotrophic factors is an attractive alternative and may be required in combination with neural transplantation as a means of providing trophic support to the grafted cells.

Another potential cell source is immortalised cell lines, the neurally committed lines, such as the Ntera2 cell line, RN33B and Hib5. Functional benefit has been reported using these cells in various animal models (Lundberg et al., 1996a; Saporta et al., 2001; Miyazono et al., 1995; Catapano et al., 1999). The Ntera2 cell line has been the most widely used. These cells are derived from human embryonal carcinomas and are terminally differentiated *in vitro* with retinoic acid. They have been found to respond to environmental cues when transplanted into the excitotoxically lesioned striatum (Saporta et al., 2001; Miyazono et al., 1995), sending out target-specific projections as well as expressing a site-specific phenotype. Grafting Ntera2 cells into the excitotoxic lesioned striatum resulted in neuronal differentiation, and a preliminary study reported rather dramatic functional effects (Hurlbert et al., 1999). However, on more detailed analysis the cells did not express any striatal-specific markers and there was no sustained improvement on skilled paw reaching and cylinder placing (Fricker-Gates et al., 2004a). Transplantation of the RN33B cell line to the lesioned and non-lesioned striatum of rats has demonstrated their potential to differentiate into neurons

in a site-specific way and form connections with target areas such as the globus pallidus (Lundberg et al., 1996b), although only a proportion of the cells showed this differentiation potential. A major disadvantage of using such cell lines is the genotypic variability that arises from the immortalization process (Renfranz PJ et al., 1991), and the risk that cells continue to proliferate to form tumours after transplantation.

1.8 Conclusions

The replacement and repair of striatal neurons by transplantation in Huntington's disease may achieve circuit reconstruction and might alleviate some of the devastating symptoms associated with the disease. Transplants using primary foetal striatum as the donor tissue are crucial for proof of principle, but ethically and logistically this donor source will not be suitable for widespread therapeutic application. Alternative cell sources including xenografts, engineered cell lines and stem cells may have the potential to replace primary foetal tissue and hence to provide a cell source that would be widely available to patients. Neural transplantation may not be a suitable therapy for all patients and the degree of degeneration may be one limiting factor. The extent to which the disease has progressed, and its relationship to transplant survival are issues that are as yet unanswered. Promising results of the first clinical trials make this an exciting field worthy of active and focussed further investigation.

Chapter Two

Materials and Methods

In vitro methods

2.1 Cell Culture

Embryos (E14) were collected in Hanks solution over ice. On removal of the head (Figure 2.1) the cortical and striatal eminencies were dissected according to (Dunnett, 1996) (Figure 2.2). Dissection was undertaken using a dissecting microscope in a laminar flow hood. Dissected tissue was collected using a pasteur pipette and left to settle in a 15ml tube containing Hanks solution on ice. The medium was removed and 200µl of trypsin/DNAse was added to the tissue for 20 minutes at 37°C. Trypsin inhibitor and DNAse were added, mixed and incubated for 5 minutes at 37°C. The tissue was then washed twice with normal medium, Dulbecco's modified Eagle's medium (DMEN F-12) supplemented with 1%PSF, and then collected by centrifugation at 1000rpm for 3 minutes. The medium was poured off and the tissue was resuspended in normal medium plus growth factors at (FGF-2, 20ng/ml and EGF, 20ng/ml), proliferation medium. The tissue was resuspended in 200µl normal medium and then triturated using a fire-polished pipette or no more than 10 strokes of a 200µl Gilson pipette to produce a single cell suspension. Cells were counted under a haemocytometer using trypan blue exclusion to assess the viability of the cells. To do this 10µl of the cell suspension was diluted in 40µl of differentiation medium

(DMEM/F12-1%PSF, 2%B27 and 1%fetal calf serum (FCS)), 10µl of this was mixed with an equal volume of trypan blue and finally 10µl was transferred to the haemocytometer for counting. Cell viability was calculated according to the formula:

cells/square x dilution factor x 10 = cells/µl cell suspension.

Having assessed the viability of the cells adjustments were made accordingly so as to culture the cells at a concentration of 200 cells/µl in T25 flasks with 10mls of proliferation medium (normal media plus growth factors). Cultures were maintained at 37°C in humidified 5% CO_2, 95% atmospheric air.

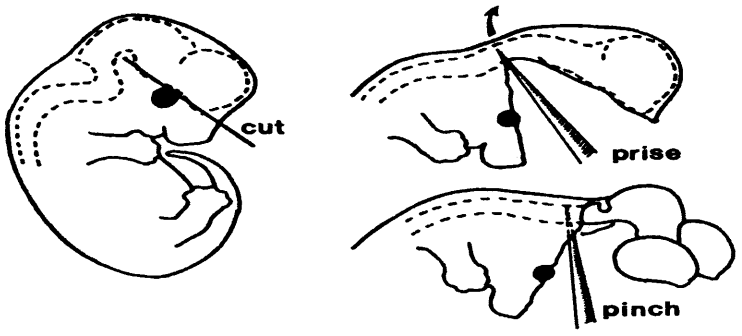


Figure 2.1 Removal of the brain from the embryo

A single cut is made just above the eye at base of the brain back to the ventral mesencephalic flexure. Using a fine forceps placed under the skin the skin and meninges overlying the whole of the brain are pulled gently away (Adapted from(Dunnett, 1996b).

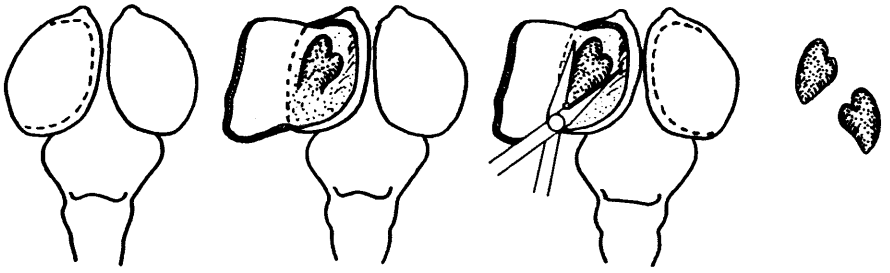


Figure 2.2 Dissection of the striatal and cortical eminencies

A longitudinal cut is made through the medial cortex, thus exposing the striatum on the floor of the lateral ventricle. Using an iridectomy scissors the striatum can be gently removed from both hemispheres. Cortical tissue is collected by carefully removing the overlying meninges (Adapted from (Dunnett, 1996b).

2.2 Human tissue collection

Human foetal tissue was collected by ultrasound guided low pressure aspiration at routine surgical termination of pregnancy (TOP), following local research ethics committee approval and under the guidelines of the Polkinghorne report (Polkinghorne, 1989) and the UK Department of Health (Department of Health, 1995), with full consent from the maternal donor, under the South Wales initiative for transplantation in HD (SWIFT-HD) program. Foetal tissue ranged in age from 5-11 weeks post conception and foetal age was based on ultrasound recordings as well as foetal morphometric measurements and using a mathematical model (Dunnett, 1996a). Embryonic age was estimated and compared against the estimated age given by the ultrasound (Figure 2.3). A previous report by our group demonstrated the value of abdominal ultrasound and low-pressure suction (Rosser et al., 2003). Using a vaginal ultrasound guidance method we have found more consistent retrieval of brain tissue. We have also demonstrated a better correlation between morphometric measurements and ultrasound estimation of gestational age using this method, R-squared value of 0.847 (previous correlation R-squared value of 0.638). This may be due to a combination of more-intact fetal tissue using this method but advances in ultrasound technology over this time period may also have contributed. Tissue was collected in hanks balanced salt solution with heparin (5ng/ml) in a 50ml tube. On removal of placenta the tissue was placed in 2mls of hibernation media for transport to the lab for dissection under a sterile laminar flow hood. Tissue of interest was carefully dissected based on the approval of at least one other experienced scientist as the foetal tissue was generally fragmented making accurate dissection difficult. Figure 2.4 shows the relative frequency of retrieving CNS tissue from specific brain regions and Figure 2.5 relates retrieval to the gestational age of the foetus.

Following dissection the tissue was prepared for ENP culture as described above for rodent tissue. One variation being that leukaemia inhibitory factor (LIF, 10ng/ml) was added to the proliferation medium.

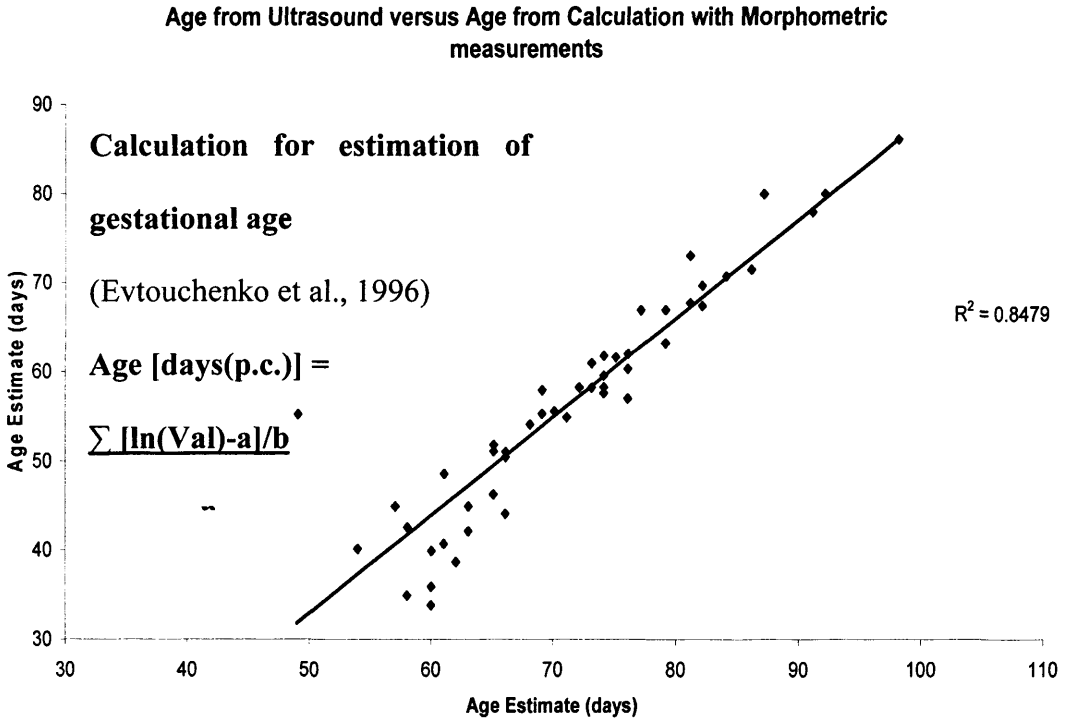


Figure 2.3 Data collected between October 2003 and May 2005 from the Cardiff TOP clinic. The x-axis gives the estimation of age from the ultrasound and the y-axis gives the estimation of age following the use of the mathematical model using morphometric measurements. There is a good, positive correlation between the two age-determining methods, with an R-squared value of 0.8479.

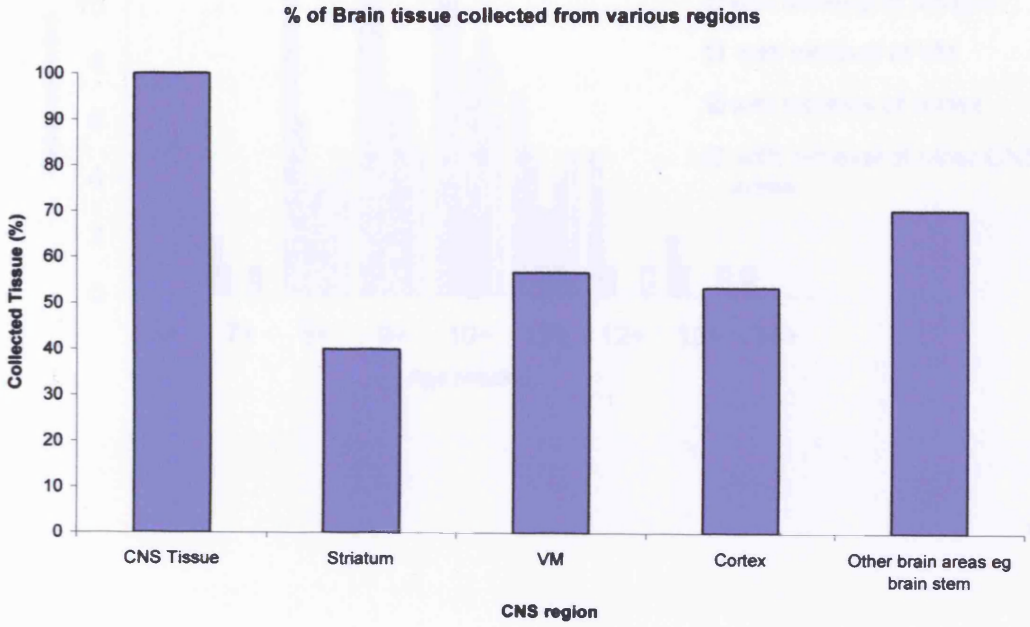


Figure 2.4. This bar chart shows the various regions of the brain that were yielded from collection and successfully dissected, as a percentage of the total CNS tissue retrieved.

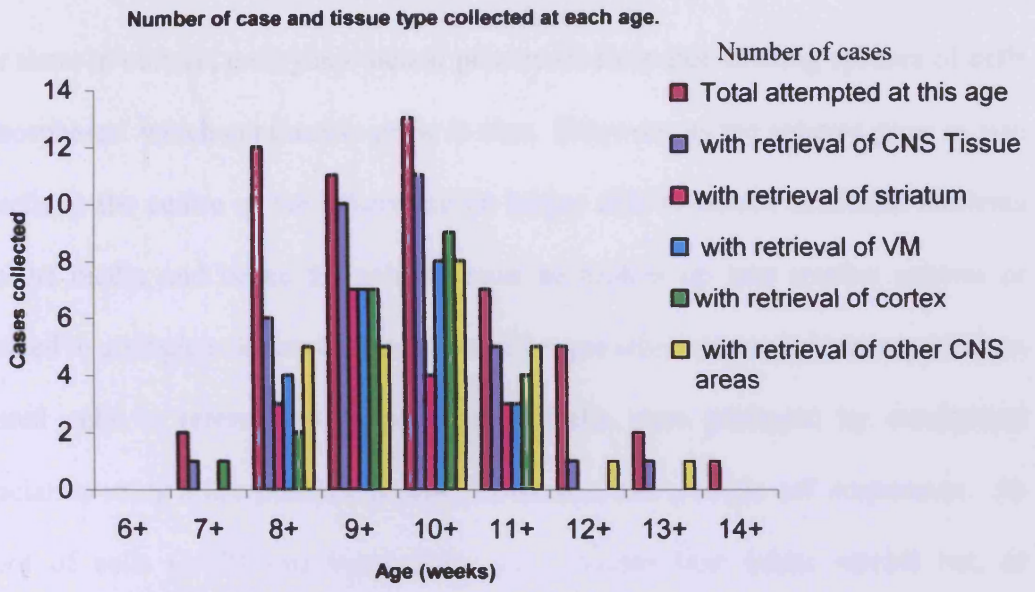


Figure 2.5. This bar chart shows the various regions of the brain retrieved at the different foetal ages collected (using ultrasound estimated age). With just the limited number of cases attempted, most success appears to be at foetal age of 9 weeks.

2.3 Passaging of cultures

Over time, in culture, embryonic neural precursors form free floating spheres of cells 'neurospheres' which continue to grow in size. However, as the spheres grow in size the cells at the centre of the sphere are no longer able to obtain sufficient nutrients from the media and hence the spheres must be broken up into smaller spheres or triturated to a single cell suspension. This technique which is carried out regularly on cultured cells is referred to as passaging. Cells were passaged by mechanical dissociation using a fire-polished pipette, 10 strokes, into a single cell suspension. An aliquot of cells (1/10) was trypsinised and a trypan blue count carried out, as described above. From this aliquot the cells were plated onto poly-L-lysine coated coverslips in 24 well plates at a concentration of 100,000 cells/30μl in the presence of differentiation medium (normal medium plus 5% foetal calf serum, FCS). 30μl of the cell suspension was plated onto the coated coverslips. Cells usually began to adhere within a few hours but in general cells were allowed to adhere over night, after which the wells were flooded with 500μl of differentiation medium per well. Cultures were maintained in the same conditions as described above for 7 days. Dissociated cells were resuspended at the same concentration as mentioned above in proliferation medium, except that B27 was replaced with 1%N2 after the first passage. Passaging was repeated every 7 days in the case of mouse tissue and every 14 days with human tissue or as required. Every 2-3 days cells were fed by replacing half the medium with fresh medium that was made up of twice the concentration of growth factors, thus maintaining the growth factor concentrations throughout.

2.4 Immunocytochemistry

Those cells that had been subjected to differentiation medium for 7 days were fixed by removing the differentiation medium and exposing the cells to a 3 minute wash in PBS (see Appendix 1). Coverslips were then subjected to 20 minutes in 4% paraformaldehyde solution at 4°C followed by 3 x 3 min, PBS. Coverslips were rinsed with 100% ethanol for 2 mins again followed by 3 x 3 min, PBS.

Indirect, single- or multiple-label, fluorescent immunocytochemistry was performed using established protocols at room temperature, except where stated (Johnstone, Turner, 1997). Appendix 2 and 3 lists the antibodies that have been used, their suppliers, whether cell permeablisation is required, the optimal determined concentration and the appropriate blocking serum/secondary antibodies.

Non-specific antibody binding was prevented by the addition of a blocking serum (isolated from a species different to that in which primary antibodies were raised) for 1hour (3% normal serum and 3%BSA in PBS). Triton X100 was omitted from this and subsequent stages of the procedure when the antigens of interest were present on the cell surface. The appropriate primary antibodies diluted in 3%BSA and 1% normal serum in PBS, were added for either 2-3 hours at room temperature or left overnight at 4°C. Removal of primary antibody was followed by 3 x 5 min, washes in PBS. In double labelling experiments, both primary antibodies (either raised in separate species, or monoclonal antibodies of different isotypes) were added contemporaneously. Secondary antibodies, (all diluted 1:200 in PBS with 1% normal serum), either directly conjugated to a fluorochrome, or, biotinylated, were then added

for 2hrs. From this point, coverslips were protected from ambient light by covering them in aluminium foil. Nuclear staining was done using the stain Hoechst; this step involved 3x5min, washes in PBS after removal of the secondary antibodies and the 4.5mins, in Hoechst (12.5µl in 10ml PBS). Again coverslips were rinsed 3 times in PBS and were then mounted onto gelatinised slides using PBS/glycerol, 1:1 as the mountant. Coverslips were sealed with clear nail varnish and stored in the dark at 4°C. Staining controls: for each primary antibody used, negative controls were run routinely and consisted of the omission of the primary antibody from the procedure.

2.4.1 BromodeoxyUridine (BrdU) immunocytochemistry

The thymine analogue BrdU is incorporated into the DNA of dividing cells in the S-phase of the cell cycle. Effective immunocytochemistry requires nuclear permeablisation and DNA denaturation to allow antibody success. Subsequent to formalin fixation, and prior to application of the blocking serum, coverslips went through a number of processing steps to accomplish this. Coverslips were post-fixed in ice-cold methanol for 20 min at -20°C, washed (3x5 min, PBS), and incubated in 2M $HCL_{(aq)}$ for 20 min at 37°C. Coverslips were then washed twice in 0.1M $NA_2B_4O_{7(aq)}$ to neutralise the acid, and washed again in PBS (3x5 min). Subsequent immunocytochemical procedures were identical to those for other primary antibodies, save that when BrdU- staining was performed in combination with labelling for another marker the entire BrdU staining protocol was completed prior to commencing staining for the other marker.

2.5 Quantification and photomicroscopy of fluorescent staining

Fluorescent staining was visualised using a Leica DRMBE microscope. The wavelengths used to visualise each stain were 560nm (red), 494nm (green) and 346nm (blue). Fluorescent tags were routinely used such that neurones stained red, astrocytes green and nuclei blue. Cell counts were taken at 40x magnification using a counting grid. In order to be as unbiased as possible, 5 random fields were chosen to take counts from. On almost all occasions, unless otherwise stated, there were 4 replicate coverslips of each condition. Colour images were processed using Optronics magnaFIRE software, and were subsequently processed using Adobe Photoshop.

2.6 Freezing Cells

When preparing tissue for grafting (see below), cells for freezing were placed in T25 flasks in complete medium overnight. Following this cells were spun at 700rpm for 6 minutes and the supernatant removed. Cells were then placed in 0.5ml freezing medium (Appendix A) and then alliquoted into cryovials at a concentration of 2 million cells per vial. A cryochamber was used to gradually bring the cells down to –80°C for subsequent storage at -80°C.

2.7 Thawing Cells

For each vial thawed, 10mls of normal medium was prepared in 15ml tubes. Cells were thawed rapidly by swirling the vial in a 37°C water bath until the contents were almost thawed. The vial was wiped with 70% ethanol and the contents quickly transferred to the 15ml tube that was spun at 1000rpm for 2-3 minutes. The

supernatant was removed and the cells were placed in 5 ml of proliferation medium before being reseeded in T25 flasks in the appropriate amount of medium.

2.8 Tissue preparation for grafting

For cells used for transplantation, the following procedure was carried out; cells were removed from flasks and an aliquot was taken for trypsinisation, trypan blue analysis, and subsequent differentiation. The remaining cells were centrifuged at 1000rpm for 3min. The clump of cells was then transferred to a sterile eppendorff tube in DNAse/glucose (10µl 0.6%glucose per 1ml DNAse). The cell suspension was centrifuged at 1000rpm for 1 min. The medium was removed and the cell concentration adjusted appropriately, based on the results from the trypan blue counts to allow for a graft of 125,000cells/µl. Cell suspension was maintained on ice until ready for grafting. The same preparation protocol was used for primary cultures.

2.9 LacZ treatment of cells

Prior to grafting cells were infected with the lentivirus LacZ. The optimum infection method was found to be 2 molecules of infection (MOI) per cell for 1 hour. To optimise the infection rate of the cells a range of MOIs were tested from very low to very high (1-80) and over various amounts of time (1-12hours). MOI of 2 for 1hr was chosen as this resulted in good cell survival as well as optimum infection of the cells. After infection, the cells were washed 3 times and resuspended for grafting based on a revised cell count.

In vivo methods

2.10 Animal care, immunosupression and anaesthesia

All animal experiments were performed in full compliance with local ethical guidelines and approved animal care according to the U.K. Animals (Scientific Procedures) Act 1986 and its subsequent amendments. Adult female Sprague-Dawley rats typically weighing 200-250g at the start of the experiments were used. They were housed in cages of 4 in a natural light-dark cycle with access to food and water *ad libitum.*

In most transplantation experiments, animals were immunosuppressed with daily intraperitoneal injections of Cyclosporin A (CsA, Sandimmun, 10mg/kg) for the duration of the experiment, commencing the day prior to transplantation. Antibacterial prophylaxis was administered by addition of aureomycin to the drinking water (5g/l) with sucrose (5g/l) and sodium chloride (0.5g/l) throughout the course of the experiment.

All surgery was performed under isoflurane anaesthesia. Anaesthesia was induced in an induction box with isoflurane and oxygen (4 l/min), and maintained by passive inhalation of isoflurane (1-2 l/min) and a mixture of oxygen (0.8 l/min) and nitrous oxide (0.4 l/min). Animals were allowed to recover in a warmed recovery chamber and received analgesia through paracetamol dissolved in drinking water (2mg/ml) for 3 days subsequent to surgery.

2.11 Quinolinic Acid Lesioning of the Rat Striatum

Animals received a unilateral 45 nmol quinolinic acid lesion of the right striatum. Quinolinic acid (QA) was dissolved in 0.1M phosphate-buffer at 15 mg/ml (90mM), and small amounts of 10M $NaOH_{(aq)}$ were added in order to fully dissolve the QA and final pH adjusted to 7.4 with concentrated $HCL_{(aq)}$. 0.75µl of solution was infused to two sites over a 3 min period using a 1µl Hamilton syringe targeted at stereotaxic coordinates: –3.2/-2.4mm lateral (L) of bregma, +0.4/+1.4 mm anterior (A) of bregma and –5.0/-4.5 mm below dura (vertical, V) with the incisor bar set to 2.3 mm below the interaural line. The syringe needle was left *in situ* for 3 min following the infusion. It was then withdrawn and the wound cleaned and sutured. For quinolinic acid lesions of the mouse striatum one infusion site was used with injection of 0.5µl over a 4 minute period targeted at the stereotaxic coordinates: -2.8mm lateral (L) of bregma, +1.0mm anterior (A) of bregma and -4.5mm below dura (vertical, V) with the incisor bar set to 0 mm below the interaural line.

2.12 Grafting tissue

Quasi-single cell suspensions of primary tissue or undissected ENP spheres were transplanted in the studies presented here. For ENPs, an estimate of cell number and viability within the ENP sphere suspensions was obtained from two representative aliquots of the suspension that were dissociated, via a trypsin digest and mechanical trituration, to single cells and counted with a haemocytometer using trypan blue exclusion. Representative aliquots were obtained by taking 1/10 of the cell sample from each flask. Using this information, undissociated spheres were harvested by centrifugation and re-suspended in DNase. Methods used to prepare primary tissue

transplantation were identical to those used to prepare such tissue for cell culture, and these cells were similarly re-suspended at a specified density in DMEM for transplantation.

In all transplant experiments into adult rat brain, 2µl of the grafting suspension were delivered slowly over a 2min period at each height using stereotaxic apparatus and a 10µl Hamilton microsyringe with a bore diameter of 0.25mm. All grafts were placed ipsilateral to the side of the lesion and were targeted to the striatum (A +1.0 mm, L – 2.8 mm, V –5.0/-4.5 mm, incisor bar set 2.3 mm below the interaural line). The needle was left *in situ* for 3 min following grafting to minimise reflux of grafted cells along the needle tract, following which the needle was removed and the skin incision cleaned, closed, and sutured. For transplants into adult mouse brain 2µl of the graft suspension were deposited to the lesioned striatum at the coordinates: A +1.2mm, L -2.6mm, V -4.5mm, incisor bar set to 0mm below the interaural line, with infusion and completion of the procedure as above.

For neonatal transplants into the rat hippocampus, at P0, the following coordinates are taken: (L) +2 mm, (A) +1mm with these points taken from the post-sagittal sinus. Hippocampal grafts into 6week old rats were at the following coordinates from bregma: L +3.5mm, A –5.5mm and (V) –2.9mm. For hippocampal grafts both at P0 and 6weeks, 2.5µl of the cell suspension were infused slowly over a 2 min period, after which time the needle was left in place for a further 1 min to prevent a back surge of the graft tissue. For neonatal rat striatal grafts coordinates used from bregma were L –1.8mm, AP +0.9mm and V –2.7mm. In this case, cells were infused over 2.5

mins and the needle was kept in place for a further 2 min. Sutured animals were administered the appropriate amount of glucose saline to prevent dehydration, for rat 5 ml, and for mouse 0.5ml.

For intraperitoneal injections (i.p.) the cell suspension was prepared as described and 1µl of cell suspension was injected over 1minute and the needle left in place for a further 1 minute. In each case 200,000 cells were injected.

2.13 Iontophoresis

Injection of the anterograde tracers neurobiotin and PHA-L was carried out using the technique of iontophoresis. This involves a current driving the tracer into the cell whilst the animal is anaesthetised. Glass micropippetes were prepared with the tip diameter ranging from 20-50 µm and were attached to an electrode holder fixed to the stereotaxic frame. A silver wire was inserted into the filled pipette and the tracer was injected with a square wave pulse of 10 micro-amps at a rate of 7secs on 7secs off for 20 minutes. Tracers were injected to the graft area using the same coordinates as those used for grafting. The tracer was injected 7 days prior to perfusion.

2.14 Perfusions and Tissue Sectioning

Animals were terminally anaesthetised with 0.8 ml intraperitoneal injection of sodium pentobarbitol (Euthatal, 200mg/ml), prior to transcaridal perfusion. When no reflexes were present the animal was perfused. The heart was exposed by cutting the skin rostrally along the midline of the thorax and by deflecting the rib cage rostrally

following a lateral incision of both sides of the cage. A spencerwells forceps blocked the main descending artery and a cannula was inserted into the left ventricle of the heart and passed into the ascending aorta. The right atrium was then incised to allow the perfusates to drain. Vascular rinse was administered for 2 min followed by 3 mins of 4% paraformaldehyde (PFA) using gravity as the pump system. The animal's head was removed using a guillotine and the brain carefully removed. Brains were post-fixed in 4% PFA for 4 hours, cryoprotected by transferring to 25% sucrose (in vascular rinse, pH 7.3) until they sank, and were then sectioned at 40μm on a freezing stage microtome. Sections were stored in 96 well plates in Tris-buffered saline (TBS) with 0.2% sodium azide added to prevent deterioration.

2.15 Cresyl Violet Staining

Cresyl violet stains nissl granules, allowing identification of different neural cell types. A 1 in 6 series of sections was mounted onto glass microscopoe slides (coated with 1% gelatin), and air-dried overnight at 37°C. Cresyl violet staining was automated on a Shandon tissue-processing machine that firstly dehydrated and rehydrated to water the sections in increasing levels of alcohol before delipidation in chloroform. They were then placed in the stain for 10 seconds, followed by a dehydration step in increasing concentrations of alcohol and finally the slides were placed in xylene before coverslips were applied with DPX mountant.

2.16 Haemotoxylin and Eosin

Another Nissl stain used was Haematoxylin and Eosin. As with cresyl violet the sections were dehydrated and rehydrated in increasing levels of alcohol before being

placed in xylene for 1hour. Sections were then placed in haematoxylin for 20minutes followed by acid alcohol for 3minutes and 1 minute of eosin before being dehydrated in increasing concentrations of alcohol. Finally the slides were placed in xylene before coversliping with DPX mountant.

2.17 Immunocytochemistry on Tissue Sections

Indirect immunocytochemistry was carried out on both mouse and rat sections for a variety of antigens, as listed in Appendix A. As with cresyl violet stains, and unless otherwise stated, a 1 in 6 series was processed. Quenching of endogenous peroxidase activity in 80% distilled water/10% methanol/10% H_2O_2, was for 5 mins. Sections were then washed 3 x 5mins in TBS and block was added for 1 hour (30µl/ml of the appropriate serum (as indicated in appendix A) in TBS +0.2% Triton X100 (TXTBS) (see Appendix A)). Without washing, the sections were transferred to primary antibody made up to the correct concentration in 1% serum in TXTBS over the weekend at –20°C. After the appropriate amount of time, the tissue sections were washed 3 x 10mins in TBS. A biotinylated secondary antibody at 1:200 in TBS with 1% serum is added for 3hours followed by another round of 3 x 10min washes in TBS. A dako streptavidin ABC kit made up of 5 µl/ml A and 5µl /ml B in TBS with 1% serum was added for 2 hours. After washing 3 x 10min TBS the sections were rinsed 2 x 5mins in 0.04M tris non-saline (TNS). Positive staining was visualised using diaminobenzidine (DAB, 0.5 mg/ml in TNS with 12.5 H_2O_2 at a 1 in 5 dilution). Finally the sections were washed 3 x 5min in TNS before being mounted on gelatinised slides and air-dried overnight prior to dehydration, clearing of any lipid residues and coverslipping in DPX mountant. Controls were routinely carried out in parallel and consisted of omission of the primary antibody.

2.18 Quantification and photomicroscopy of grafts.

Grafts were visualised under the Leitz DRMB light microscope. Digitised images were captured using a Hamamatsu C4752 video camera and NIH 1.55.2 image analysis software, and the area corresponding to grafted tissue in each section was traced around and measured. Graft volume was subsequently calculated to be:

$$V = \frac{\Sigma a.M}{f}$$

V = graft volume (mm^3)
a = area (mm^2)
M = section thickness (0.06/0.04 mm)
F = frequency of sampled sections (1:6)

Cell number was calculated where possible by counting all cells.

Abercrombie correction formula:

Total number of cells = F x A x M/(D+M)

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

However where cell number was too great to count each cell, stereology was used on an Olympus C.A.S.T. grid system in which cells were counted in random regions within the defined graft area. Using the following formula the total number of cells in the structure per section can be calculated:

$$n = \Sigma c \times (\Sigma A/\Sigma a) \times (M/d)$$

Σc = The total number of cells counted
ΣA = The sum of all the inclusion areas
Σa = The sum of all the sample areas
M = Mean section thickenss

The total number of cells in the structure is

$\sum n \times f$ **f** = the frequency of sectioning.

2.19 Statistical analyses

Genstat 5 release 3.2 (Rothampsted Agricultural Research Station, UK) package was used for parametric uni- and multifactorial analysis of variance (ANOVA), with appropriate *post-hoc* tests (Newman Keuls) performed by hand with reference to statistical tables.

Chapter 3

In vitro culture conditions: optimising growth factor concentrations for the proliferation and neuronal differentiation of ENPs.

Summary

FGF-2 and EGF are the two major mitogens for ENP proliferation in culture however, as well as their role in precursor cell expansion; FGF-2 and EGF also play a key role in the division of astrocytes, and in neuronal differentiation. Thus, it is important to establish the optimal concentrations of these factors for expansion and differentiation of neuronal phenotypes. In this chapter I explore the effect of FGF-2 and EGF concentrations ranging from 1-20ng/ml on the expansion and differentiation capacity of ENPs isolated from the striatum and cortex of E14 mice. ENP expansion was seen under all conditions, but proliferation was greatest at 10 and 20ng/ml and least at 1ng/ml. The proportion of neurons (as a proportion of total cell number) differentiating from ENP populations appeared to be greatest at 1ng/ml. However, once adjustments were made for the amount of expansion at each dose, final neuronal yield was maximal at the highest concentration of FGF-2 and EGF used.

3.1 Introduction

ENPs can proliferate in culture in the presence of mitogens and following mitogen withdrawal, ENPs spontaneously differentiate *in vitro* into the major cell types of the central nervous system. Both the proliferation and differentiation of ENPs is influenced by the presence of mitogens in the culture medium (Tropepe et al., 1999), and growth factors have been shown to act in a sequential manner in the regulation of differentiation (Vescovi et al., 1993; (Ciccolini and Svendsen, 1998). A number of growth factors have been explored, including nerve growth factor (NGF), epidermal growth factor (EGF), basic fibroblast growth factor (FGF-2), insulin like growth factor (IGF), brain-derived neurotrophic factor (BDNF) and tumor necrosis factor (TNF) (Arsenijevic et al., 2001; Arsenijevic and Weiss, 1998; Cattaneo and McKay, 1990; Murphy et al., 1990; Santa-Olla and Covarrubias, 1995; Tropepe et al., 1997). The factors important for continued proliferation of these precursors are complex, and likely to involve contact as well as diffusible elements (Svendsen et al., 1998; Temple and Davis, 1994), although it is clear that FGF-2 and EGF are important mitogens for such cells (Anchan et al., 1991; Gensburger et al., 1987; Kilpatrick and Bartlett, 1993; Lillien and Cepko, 1992; Ray et al., 1993; Reynolds and Weiss, 1992a; Vicario-Abejon et al., 1995). However, the relationship between cells grown in response to either EGF or FGF-2 is unclear; comparisons between published reports are difficult because of variations in embryonic ages, growth factor combinations, and the brain regions used. To add to this complex situation, both EGF and FGF-2 can stimulate the division of astrocytes as well as neurons (Hou et al., 1995; Kilpatrick and Bartlett, 1995) which are themselves capable of making and releasing a wide range of growth factors.

A further important complexity is the observation that there may be significant species differences in the behaviour of ENPs (Smith et al., 2003). For example, EGF responsive neural precursor cells isolated from the embryonic mouse brain expand for long periods of time whilst retaining pluripotency (defined by their ability to produce neurons, astrocytes and oligodendrocytes) in contrast to EGF responsive cells from the developing rat striatum that only undergo a finite number of divisions before entering crisis (Svendsen et al., 1997b). It has also been shown that FGF-2 responsive neural precursors isolated from E14 mouse striatum arise earlier than EGF responsive cells during development *in vitro* (Ciccolini and Svendsen, 1998), a result confirmed using mouse striatum, by isolating precursors from different gestational ages (Tropepe et al., 1999). Ciccolini (Ciccolini, 2001) has also reported that there is a developmental switch in the responsiveness of ENPs from FGF-2 to EGF both *in vitro* and *in vivo*. Furthermore, early mouse precursors (E14) are responsive to FGF-2 but by E18 these cells are responsive to both FGF-2 and EGF (Ciccolini, 2001).

Both FGF-2 and EGF are considered to act on the classical receptor tyrosine kinases that induce the activation of the Ras/extracellular signal-regulated kinase (ERK) pathway that ultimately leads to the phosphorylation of the transcription factor CREB (Marshall, 1995). In the absence of mitogen stimulation only 1% of cells show nuclear phospho-CREB immunoreactivity whereas in the presence of EGF and FGF-2 or FGF-2 alone 10-12% of cells are immunoreactive for CREB phosphorylation, in E14 striatal cultures 24hrs after culturing (Ciccolini and Svendsen, 1998). FGF-2 is used in the presence of the stabilising agent heparin which has been shown to enhance FGF-2 induced proliferation whilst also reducing the numbers of astrocytes produced on differentiation (Caldwell and Svendsen, 1998; Caldwell et al., 2004). Insulin is

present (at a concentration of 500μg/ml) and has also been found to enhance the proliferative effects of FGF-2, predominantly via the type 1 IGF receptor (Drago et al., 1991; Santa-Olla and Covarrubias, 1999).

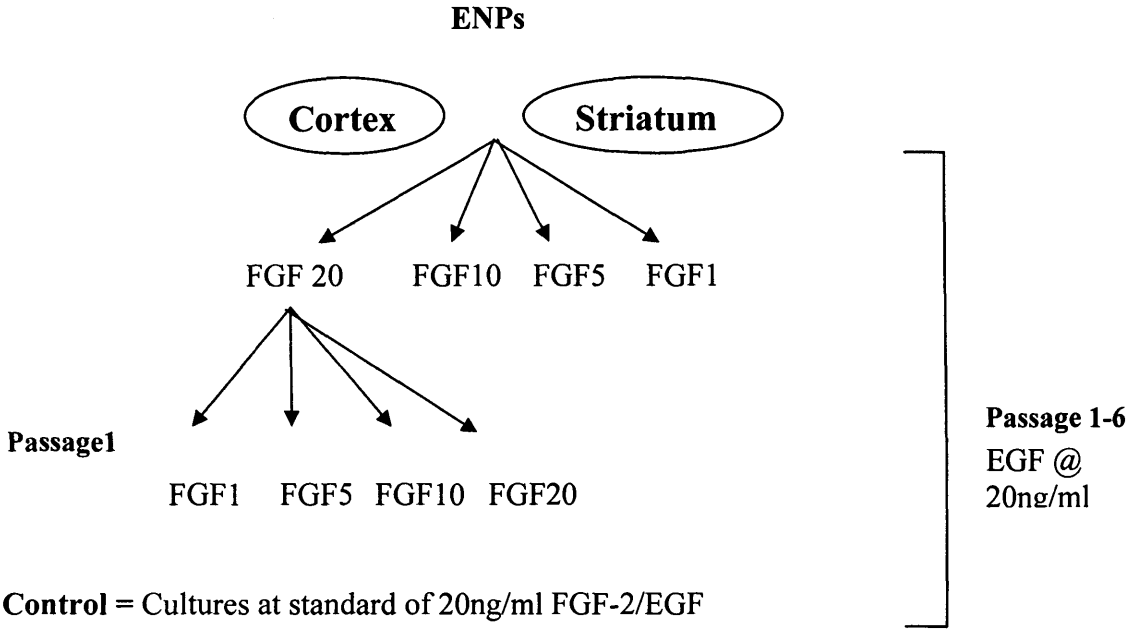
Here we report the effects of a range of FGF-2 and separately, EGF concentrations on the proliferation of E14 mouse-derived striatal and cortical ENPs, and the capacity of these cells to differentiate along a neuronal phenotype *in vitro* following mitogen withdrawal. Two separate experiments were carried out, one looking at the effects of varying FGF-2 concentrations, experiment 1a, and the other the effects of similar concentrations of EGF, experiment 1b. For each experiment the corresponding mitogen concentration was maintained constant at 20ng/ml, the optimal concentrations previously reported in the literature.

3.2 Experimental design

The rationale for experiment 1a was firstly to establish the optimum concentration of FGF-2 for the proliferation and, more importantly, the neuronal differentiation of striatal and cortical-derived ENPs, and secondly to establish whether priming cells with 20ng/ml of FGF-2, would allow the cells to establish and subsequently be responsive to lower concentrations. An additional group was maintained at 20ng/ml FGF-2, the most frequently used concentration in the literature, as a control. Throughout experiment 1a the concentration of EGF was maintained constant at 20ng/ml. All cultures were maintained over 6 passages and at each passage cells were plated for differentiation.

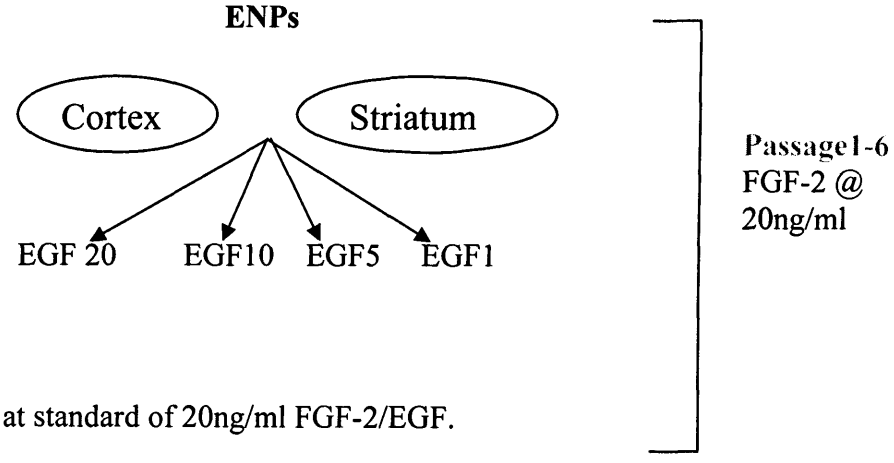
Figure 3.1A/B (overleaf). Experiment 1a was carried out such that the cells were exposed to various concentrations of FGF-2. Both striatal and cortical-derived murine E14 tissue were exposed to concentrations of 1,5,10 and 20ng/ml of FGF-2. After one week one group of cells that had been primed with 20ng/ml of FGF-2 were subsequently cultured in lower concentrations of FGF-2 for the remainder of the experiment. In experiment 1b the FGF-2 concentration was maintained at 20ng/ml. The cultures were exposed to 1,5,10 and 20ng/ml EGF over a period of 6 passages with cells being plated at each passage for differentiation.

Figure 3.1A Experiment 1a



Control = Cultures at standard of 20ng/ml FGF-2/EGF

Figure 3.1B Experiment 1b



Control = Cultures at standard of 20ng/ml FGF-2/EGF.

3.3 Results

3.3.1 ENP proliferation

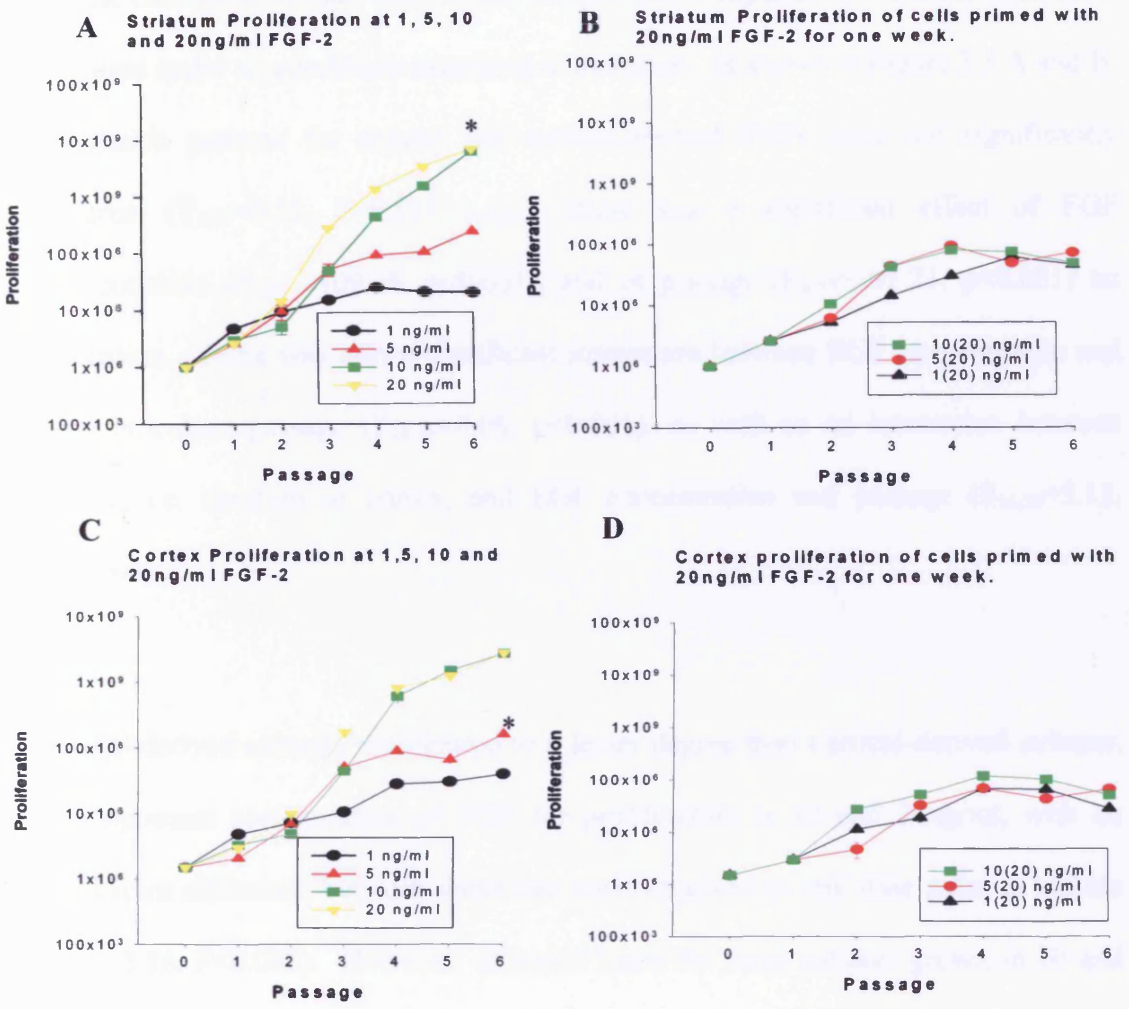
i) The effects of various concentrations of FGF-2

Mouse embryonic striatal and cortical-derived ENPs expanded in number over the 6 passages under all conditions examined in this study, as shown in Figure 3.2 A and C. Expansion profiles were between 10^2 to 10^4 fold over the 6 passages, depending on the dose of FGF-2 used. Expansion patterns for striatal and cortical-derived ENPs were not significantly different: all cultures showed a trend for an increase in expansion over time ($F_{1,35}$=1.38, P=2.4 n.s). There was a significant effect of FGF-2 concentration ($F_{6,35}$=150.98, p<0.001), and of passage ($F_{6,35}$=916.21, p<0.001) on expansion. Before passage 2 (P2), expansion rates are similar across all doses of FGF-2. After P2 differences emerge, and the greatest levels of expansion are seen at 10 and 20ng/ml FGF-2. Thus for both striatal and cortical-derived cells, 20ng/ml and 10ng/ml FGF-2 resulted in greater proliferation than did 5ng/ml FGF-2 (which produced the next highest level of proliferation): for striatal-derived tissue t_{46}=7.39, p<0.001 and t_{47}=8.26, p<0.001 for 20ng/ml and 10ng/ml vs 5ng/ml respectively; and for cortical-derived tissue t_{51}=10.39, p<0.001 and t_{52}12.26, p<0.001 for 20ng/ml and 10ng/ml vs 5ng/ml respectively.

The effect of priming cells with 20ng/ml FGF-2 for the first week followed by a reduction to 10, 5, or 1ng/ml is shown in Figure 3.2 B and D. For cultures grown in 10ng/ml FGF-2, exposure to 20ng/ml for the first week appeared to have a detrimental effect on expansion (t_5 = 6.83; p< 0.05 at P4, and t_{13} = 16.31; p< 0.001 by P6). No

significant effect of priming was seen at 1 and 5ng/ml FGF-2 with these cultures proliferating at similar rates to those maintained at 1 and 5ng/ml throughout.

Figure 3.2



Legend to Figure 3.2

Proliferation capacity of E14 Striatal and Cortical-derived cells *in vitro*. A and C) For both striatal and cortical-derived cultures, 10 and 20ng/ml FGF-2 resulted in significantly greater proliferation than did 5 and 1ng/ml (*$p<0.001$). B and D) show the proliferation of cultures primed with 20ng/ml FGF-2 for one week before being cultured at a lower concentration of 1, 5, or 10ng/ml. Primed cultures did not proliferate significantly better than those at the lower concentration throughout. Cells grown at 10ng/ml proliferated significantly less when primed with 20ng/ml FGF-2 for one week than those not primed for the first week.

ii) ENP proliferation in the presence of various concentrations of EGF

Mouse embryonic striatal and cortical-derived ENPs expanded in number over the 6 passages under all conditions examined in this study, as shown in Figure 3.3 A and B. Expansion patterns for striatal and cortical-derived ENPs were not significantly different ($F_{1,35}$=0.52, P=0.817 n.s). There was a significant effect of EGF concentration ($F_{3,35}$ =109.26, p<0.001), and of passage ($F_{6,35}$=192.21, p<0.001) on expansion. There was also a significant interaction between EGF concentration and time in culture/passage ($F_{18,35}$=8.08, p<0.001), as well as an interaction between source, i.e. striatum or cortex, and EGF concentration and passage ($F_{18,35}$=3.13, p<0.001).

Striatal-derived cultures proliferated to a lesser degree than cortical-derived cultures. The optimum concentration of EGF for proliferation is 10 and 20ng/ml, with no significant difference between these two concentrations at any time point in culture ($F_{6,35}$=5.16, P<0.005). However, at both P5 and P6 these cultures grown in 10 and 20ng/ml EGF expanded more than those maintained in 5ng/ml of EGF (the concentration resulting in the next highest proliferation): P5: t_7=5.05, p<0.001, and t_6=4.23, p<0.005; P6: t_{12}=8.23, p<0.001, and t_{11}=7.29, p<0.001 for 20ng/ml vs 5ng/ml and 10ng/ml vs 5ng/ml respectively. There was also a significant difference between cultures expanded in 5ng/ml and those in 1ng/ml EGF at P4 and P5 (P4: t_{11}=5.52, p<0.001, and P5: t_{10}=4.94, p<0.005). However this was no longer significant at P6 (t_5=3.17, n.s).

In the case of cortical-derived cultures there were differences between the higher and lower concentrations at all time points except P4 at which point there was no

difference between any of the EGF concentrations. By P6 it is apparent that cortical-derived cultures exposed to 10 or 20ng/ml EGF proliferated significantly better that those at 5ng/ml (t_8=6.46, p<0.001, t_7=6.23, p<0.001) and 1ng/ml (t_{19}=11.75, p<0.001, t_{18}=11.633, p<0.001), with there being no significant difference between 10 and 20ng/ml (t_2=0.025, ns). At P4, P5 and P6 cultures maintained in 5ng/ml proliferated significantly better than those in 1ng/ml (P4: t_{12}=6.463, p<0.001, P5: t_{15}=6.82, p<0.001, and P6; t_{12}=5.41, p<0.005).

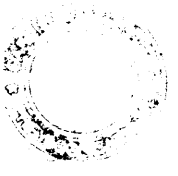
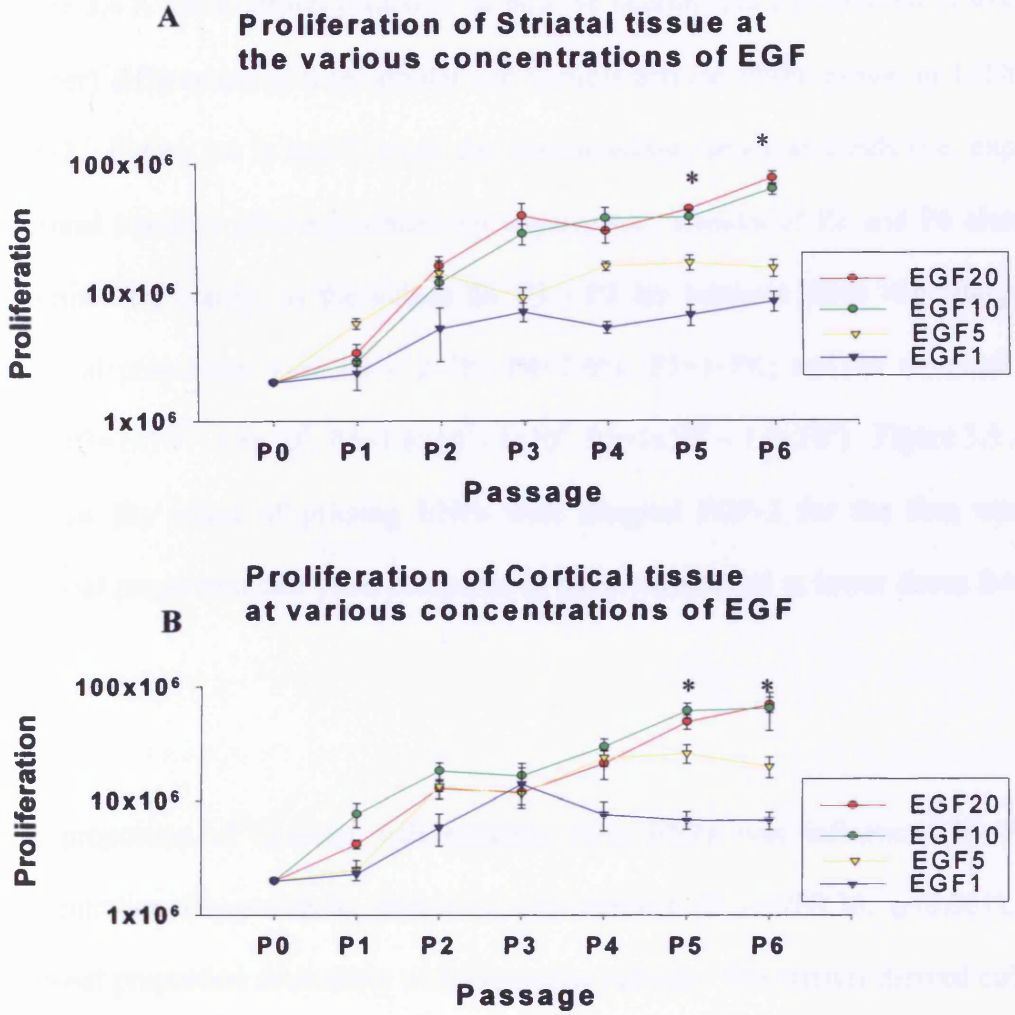


Figure 3.3 A and B



Legend to Figure 3.3 A and B.

Proliferation of striatal (A) and cortical (B)-derived cultures over a period of 6 passages in culture. In the presence of 20ng/ml FGF-2, 10 and 20ng/ml EGF, proliferation was maximum when compared with cultures expanded in 5 and 1ng/ml EGF, for striatal and cortical-derived cultures respectively. Proliferation at all lower concentrations assessed was significantly less, $p<0.001$.

3.3.2 ENP differentiation

(i) The effect of varying concentrations of FGF-2 on neuronal proportion

Figure 3.4 A and C shows β-tubulin III positive neurons (as a proportion of total cell number) differentiating from striatal and cortical-derived ENPs grown in 1-20ng/ml FGF-2. Figure 3.4 B and D show the corresponding neuronal yields (i.e. expected neuronal numbers after adjustment for expansion). Results of P2 and P6 alone are presented for clarity, as the values for P3 - P5 lay between these two (ranges for neuronal proportion were P3 = 2-7%; P4=2-6%; P5=1-5%; and for neuronal yield were P3=$1x10^2$ –$1.8x10^3$, P4=$1.6x10^2$ –$1x10^4$, P5=$1x10^3$ – $1.8x10^4$). Figure 3.5 A and B show the effect of priming ENPs with 20ng/ml FGF-2 for the first week on neuronal proportion and yield compared to those maintained at lower doses from the start.

The proportion of neurons differentiating from ENPs was influenced by FGF-2 concentration ($F_{2,18}$=119.36, $p<0.001$), and passage ($F_{1,18}$=280.38, $p<0.001$), with neuronal proportion decreasing with increasing passage. For striatal-derived cultures, the highest neuronal proportions were seen with the lower concentrations of FGF-2 (see below), although even at 1ng/ml FGF-2, the predominant cell type following differentiation is the astrocyte (Figure 3.8).

Between P2 and P6 there is a significant decline in the proportion of neurons differentiating from both striatal and cortical-derived cultures ($F_{1,18}$=280.38, $p<0.001$). For striatal-derived cultures at P2 there was a significant difference in the proportion of neurons differentiating from cultures in 1ng/ml compared to 5, 10 and 20ng/ml

(t_5=5.12, $p<0.05$, t_3=4.62, $p<0.05$, t_2=3.5, $p<0.05$). At P6 there was a significant difference between cultures at 1 and 5ng/ml (t_3=7.41, $p<0.001$), but there were no other significant differences. Post hoc analysis of the neuronal proportions for cortical-derived cultures at P2 revealed that there was no difference between cultures maintained in 1 or 10ng/ml nor between 5 and 20ng/ml, but significant difference existed between either 1 or 10ng/ml compared to 5 or 20ng/ml (t_5=4.4, $p<0.05$ and t_4=3.43, $p<0.05$). By P6 there was still no significant difference between 1 and 10ng/ml, however there was significant differences between cultures maintained in 10ng/ml compared to 5ng/ml and 10ng/ml compared to 20 ng/ml (t_2=2.63, $p<0.05$ and t_5=6.85, $p<0.001$ respectively).

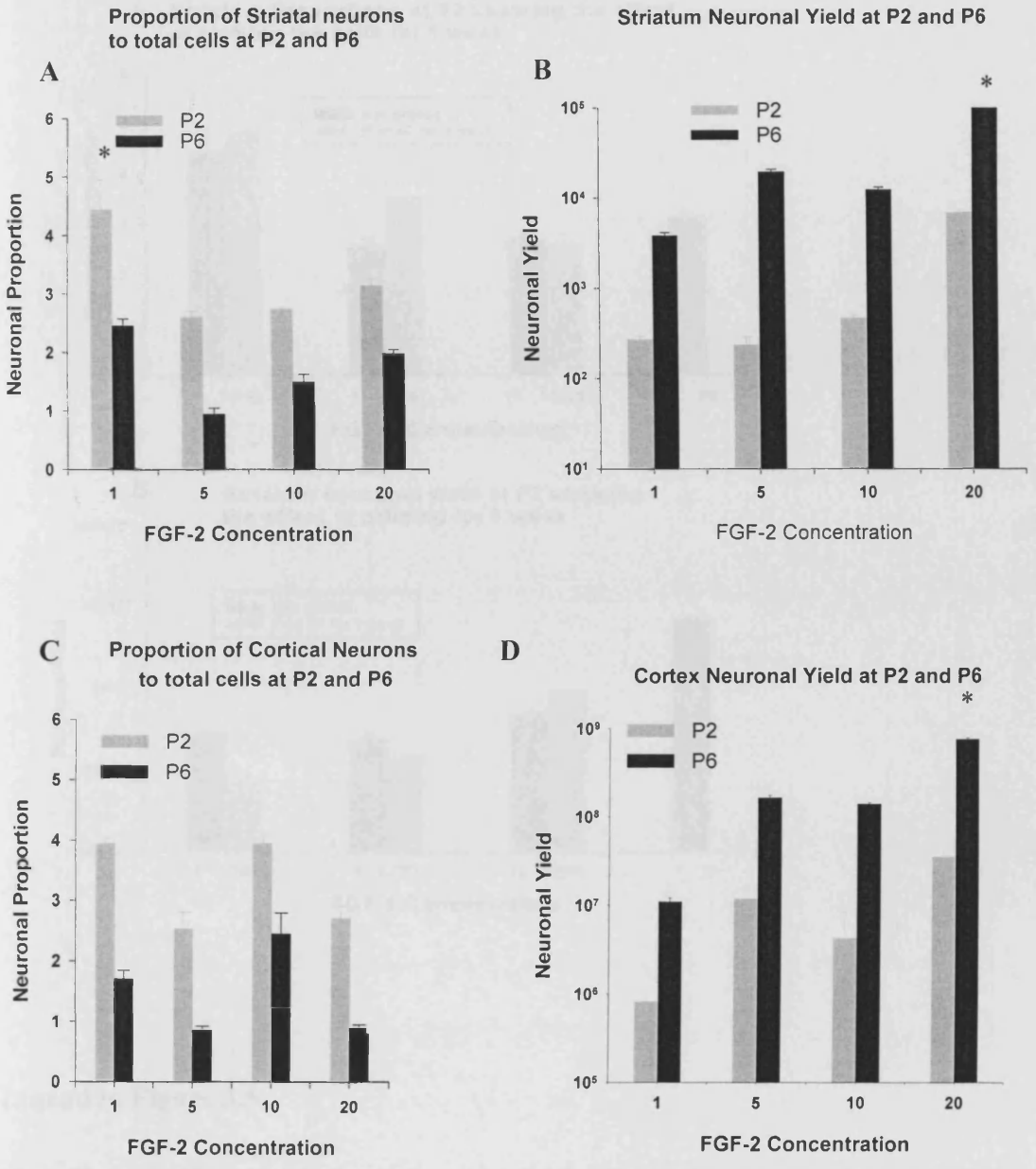
The effect on neuronal differentiation of exposing ENPs to 20ng/ml FGF-2 for the first week were similar for striatum and cortex, and thus, for clarity of presentation, only the former is shown (Figure 3.5). Following differentiation, neuronal proportion was higher if ENPs had been exposed to 20ng/ml FGF-2 for the first week of proliferation ($F_{1,18}$=11.29, $p<0.001$). This was entirely due to a significant increase in neuronal proportion in cells reduced to 5ng/ml after one weeks expansion in 20ng/ml (t_2 =6.2 $p<0.001$) and did not reach significance at any other concentration.

(ii) Neuronal yields - FGF-2

Overall, neuronal yields were greater for cortex than striatum ($F_{1,18}$=8.60, $p<0.05$). Neuronal yields were affected by FGF-2 concentration ($F_{2,18}$=51.25, $p<0.001$), but in the opposite direction to the neuronal proportion, with the greatest yields being at 20ng/ml (compared to 10ng/ml: t_5 = 7.67; $p<0.01$; 5ng/ml: t_2 = 4.07; $p<0.05$; and

1ng/ml: t_7 = 15.59; $p<0.01$) for cortical-derived cultures and (compared to 10ng/ml: t_6=8.136, $p<0.001$; 5ng/ml: t_7=11.97, $p<0.001$ and 1ng/ml: t_9=13.17, $p<0.001$), for striatal-derived cultures. Exposure to 20ng/ml FGF-2 for the first week of proliferation did not have a significant effect on yield ($F_{1,18}$=1.46 n.s Figure 3.5B).

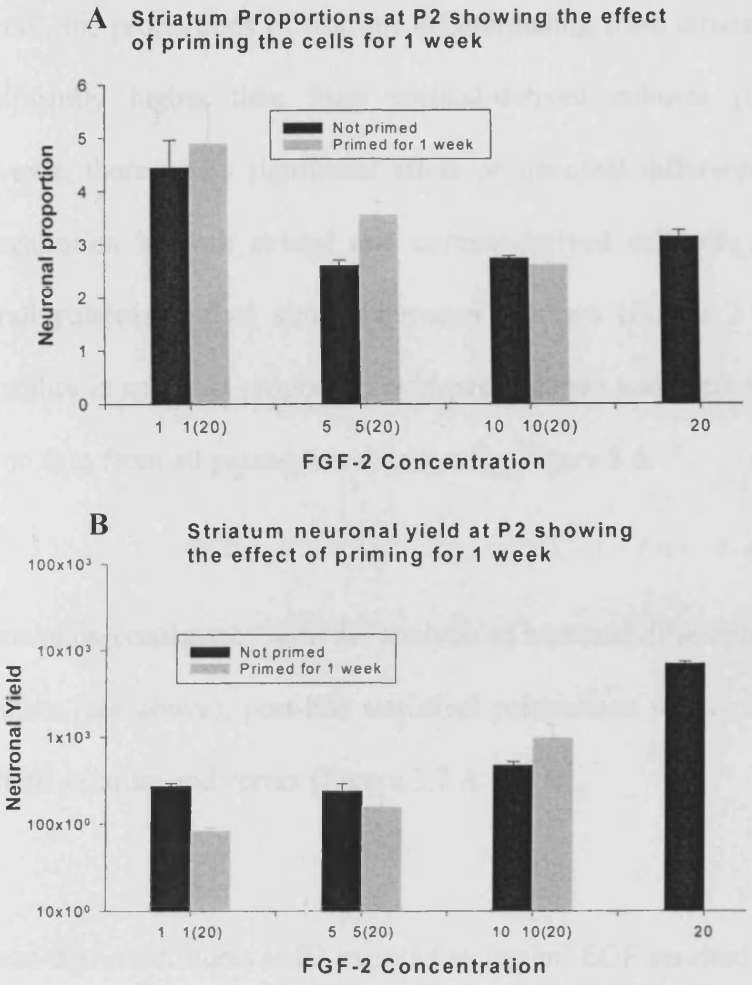
Figure 3.4 (A, B, C and D)



Legend to Figure 3.4 A,B C and D

The neuronal differentiation of E14 Striatal and Cortical-derived ENPs at 1, 5, 10, and 20ng/ml FGF-2. A and C represent the neuronal proportion of striatal and cortical-derived ENPs respectively. Following adjustment for proliferation B and D – greatest differentiation was at 20ng/nl FGF-2. (Note the axis difference in B and D)

Figure 3.5 A and B



Legend to Figure 3.5

The effect of priming cultures to 20ng/ml FGF-2 in the first week of culture before subsequently reducing the FGF-2 concentration to 1, 5 or 10ng/ml FGF-2 is compared to those cultures exposed to the same concentration of FGF-2 for the entire experiment. A) Compares the effect of priming with 20ng/ml FGF-2 on the neuronal proportions of striatal-derived cultures at P2 whereas B) compares the neuronal yields of striatal-derived cultures also at P2.

(iii) The effect of varying the concentration of EGF on neuronal proportion

Overall, the proportions of neurons differentiating from striatal-derived cultures was significantly higher than from cortical-derived cultures ($F_{1,18}$=21.72, p<0.001). However, there was a significant effect on neuronal differentiation of varying EGF concentration in both striatal and cortical-derived cells ($F_{3,18}$=4.34, p<0.001) and overall patterns looked similar between the two (Figure 3.6). There was more variability in neuronal proportion over passage than was seen with FGF-2 and for this reason data from all passages is displayed in Figure 3.6.

To maintain consistency with the analysis of neuronal differentiation following FGF-2 variation (see above), post-hoc statistical comparison was carried out for P2 and P6 for both striatum and cortex (Figure 3.7 A and B).

Striatal-derived cultures at P2 exposed to 1ng/ml EGF resulted in a greater proportion of neurons than did cultures exposed to 5, 10 and 20ng/ml EGF (t_2=15.823, p<0.001, t_4=16.609, p<0.001, t_7=22.358, p<0.001). Cultures exposed to 5 and 10ng/ml EGF also resulted in significantly greater neuronal proportions than those exposed to 20ng/ml EGF (t_4=5.248, p<0.001, t_4=5.749, p<0.001). There was no significant difference between cultures exposed to 5 and 10ng/ml EGF (t_2=0.78, NS). The pattern was similar at P6: cultures exposed to 1ng/ml EGF resulted in a greater neuronal proportion than those exposed to 10 and 20ng/ml EGF (t_4=4.204, p<0.005, and t_6=8.76, p<0.001); cultures exposed to 5 ng/ml EGF produced a greater proportion of neurons than those exposed to 20ng/ml (t_4=5.248, p<0.001); and there

were no significant difference between cultures at 1ng/ml and 5ng/ml EGF nor 5ng/ml and 10ng/ml EGF (t_3=3.15, NS 1 t_2=0.68, NS).

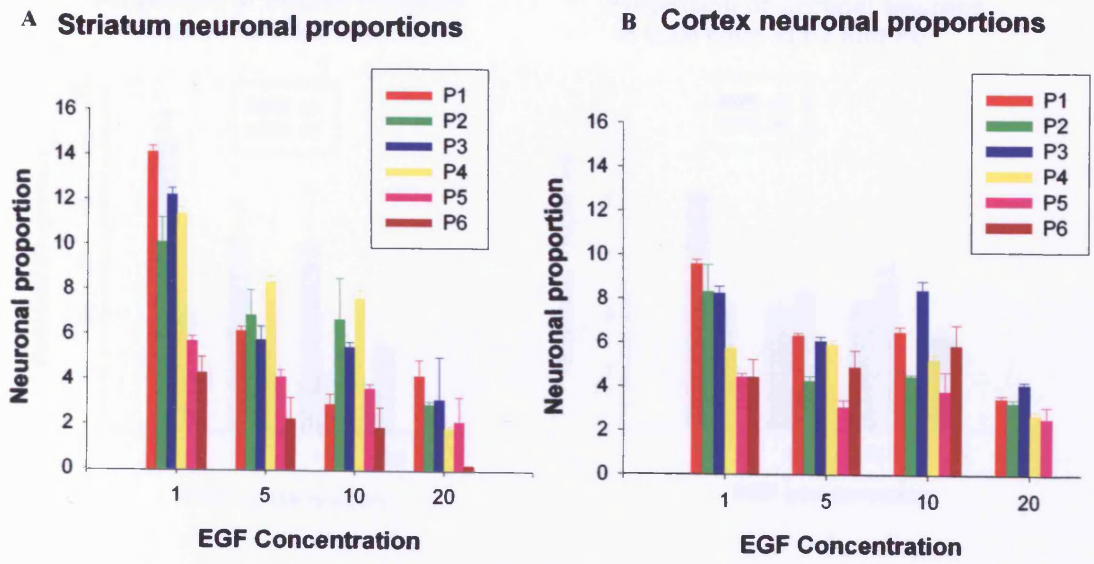
Cortical-derived cultures at P2 exposed to 1, 5, and 10ng/ml resulted in significantly greater neuronal proportions than those exposed to 20ngml (t_4=10.07, P<0.001, t_3=4.84, P<0.001, t_5=10.56, P<0.001, respectively). Cultures exposed to 1ng/ml also resulted in a significantly greater neuronal proportion than the cultures exposed to 5ng/ml and 10ng/ml (t_2=5.233, p<0.001, t_3=5.723, P<0.001, respectively). There was no significant difference between those exposed to 5 and 10ng/ml EGF (t_2=0.49, ns). At P6 differences in neuronal proportions only reached significance when comparing cultures exposed to 1ng/ml EGF with those exposed to 20ng/ml EGF which had much less neurons (t_5=4.52, P<0.005).

(iv) Neuronal yields - EGF-2

Overall there was no difference between yields for striatal and cortical-derived cells. For striatal-derived cultures, there was no effect of varying EGF concentration at P2 (F_3=4.07, ns). In contrast, at P6 cultures exposed to 5, 10 and 20ng/ml EGF showed significantly greater neuronal yields than cultures exposed to 1ng/ml EGF (t_2=6.24, p<0.001, t_3=9.43, p<0.001 and t_4=11.04, p<0.001). There was no significant difference between cultures exposed to 5, 10 and 20ng/ml EGF (Figure 3.7C).

There was no interaction between the EGF concentration, passage and the source. In the case of cortical-derived cultures there was no significant effect of EGF concentration either at P2 or P6 (Figure 3.7D).

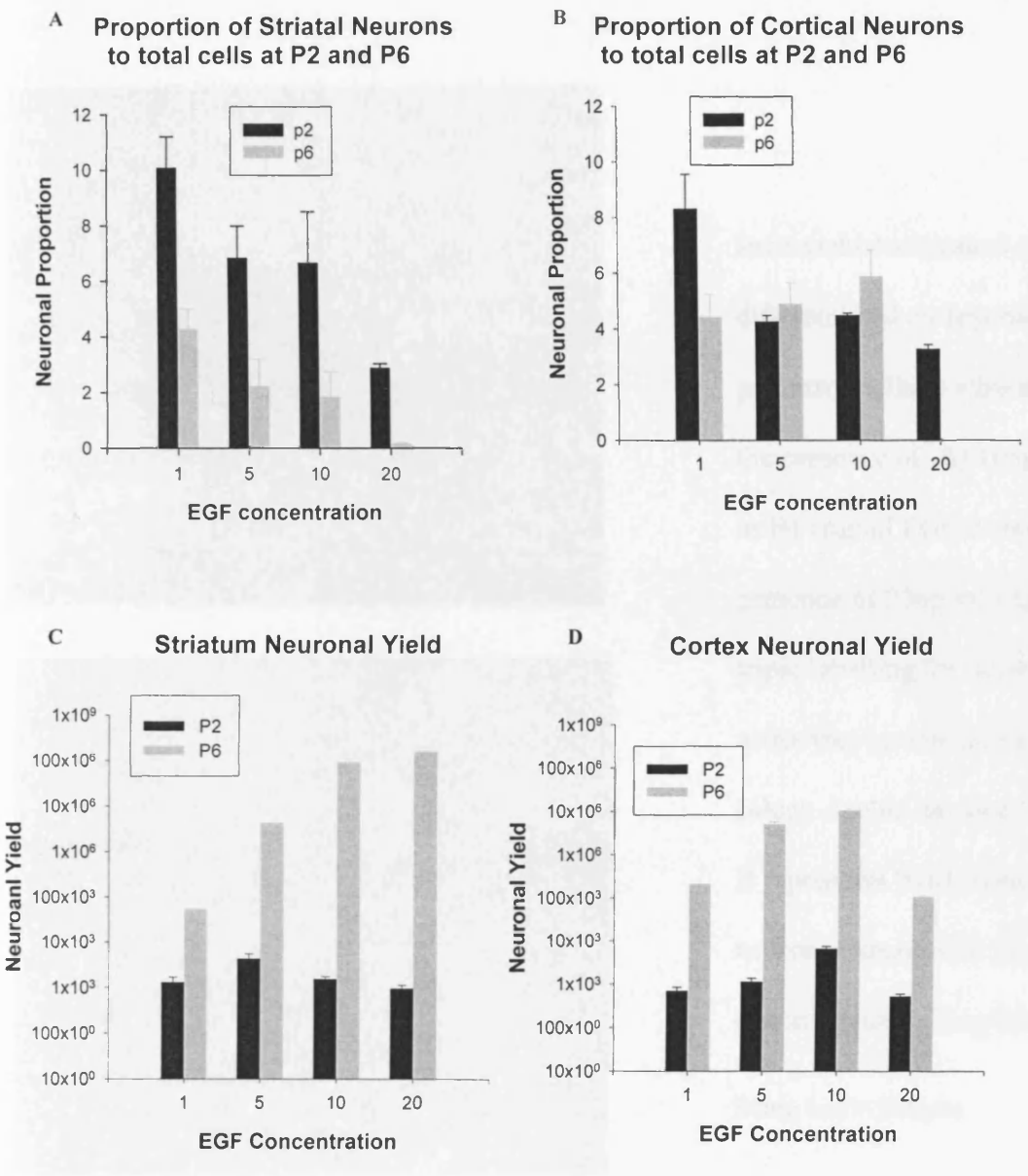
Figure 3.6 A and B



Legend to Figure 3.6

The neuronal differentiation of E14 striatal and cortical-derived ENPs at 1, 5, 10, and 20ng/ml EGF. A and B represent the neuronal proportion of striatal and cortical-derived ENPs respectively.

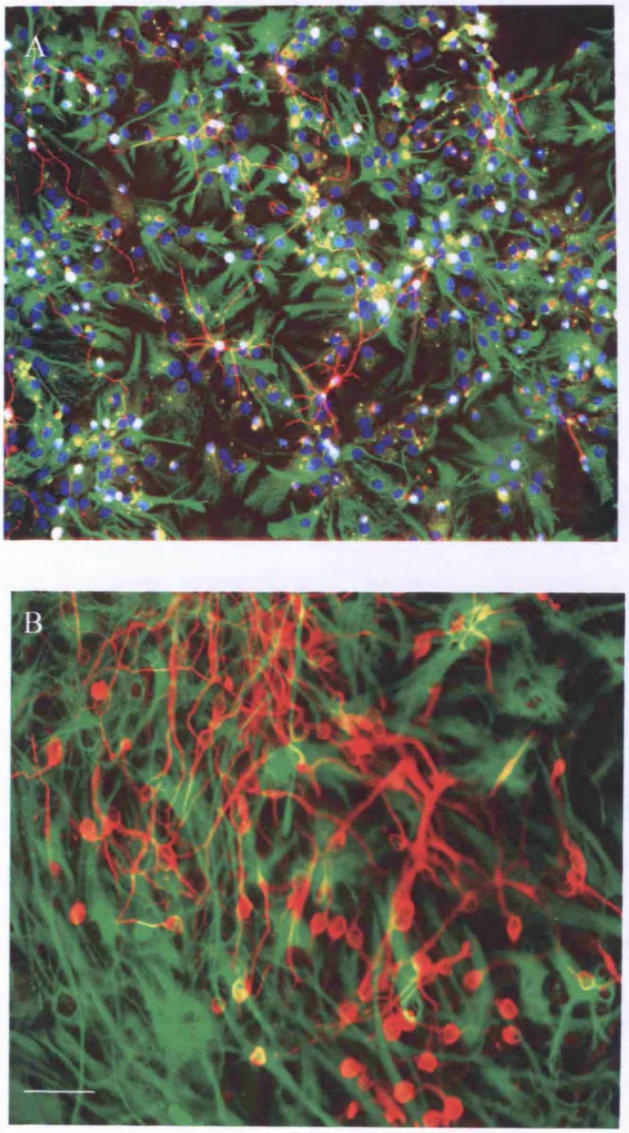
Fgure 3.7 A,B,C and D



Legend to Figure 3.7

The neuronal differentiation of E14 striatal and cortical-derived ENPs at 1, 5, 10, and 20ng/ml EGF. A and B represents the neuronal proportions for striatal and cortical-derived cultures. C and D) Following adjustment for proliferation – greatest differentiation of cortical-derived cultures was at 10ng/ml compared to 5 and 20ng/ml and for striatal-derived cultures at 5, 10 and 20ng/nl FGF-2 compared 1ng/ml.

Figure 3.8 A and B



Immunohistochemical staining of differentiated embryonic neural precursor cells *in vitro* expanded in the presence of; A) 10ng/ml FGF-2 or B) 1ng/ml FGF-2 (both in the presence of 20ng/ml EGF). A is triple labelling for neurons (red), astrocytes (green) and a nuclear stain (blue), double labelled cells (yellow). B represents B-III tubulin positive neurons expanded at the lower concentration of 1ng/ml FGF-2 at P2.

Scale bar = 200μm

3.3.3 Comparison between Experiment 1a and 1b.

Table 1 summarizes the results from both experiments 1a and 1b with the data presented as the range for each concentration of each growth factor assessed in each group. Overall the trends are similar in the two experiments although some intriguing differences are apparent. In the case of neuronal proportions there is a large difference between the experimental groups. In experiment 1a the EGF concentration was maintained constant at 20ng/ml and there was a much lower neuronal proportion whereas in the experiment 1b, in the condition where EGF concentration was low and the FGF-2 concentration was constant at 20ng/ml there was a greater proportion of neurons differentiation in these cultures. For neuronal yield which takes into account the proliferation of the cultures, there was also a big difference between the two experiments with striatal cultures in the presence of varying concentrations of EGF resulting in a greater neuronal yield than in the presence of varying concentrations of FGF-2. The reverse was true for cortical cultures in that varying FGF-2 concentrations resulted in greater neuronal yield than did varying EGF concentration. It is also important to point out that there is a difference in the neuronal differentiation between the two experiments in the case of the control cultures (20ng/ml EGF and 20ng/ml FGF-2) which highlights the variability between experiments in the expansion and neuronal differentiation of CNS derived ENPs. Thus, it is important to be cautious in interpreting these comparisons.

Summary Table

	Striatum		Cortex		
	P2	P6	P2	P6	**Comments**
Mitogen concentration range	**Proliferation (total cell number)**				
FGF-2 Low to high	10^6-10^7	10^7-10^9	10^6-10^7	10^7-10^9	No difference between Striatum and Cortex
EGF Low to high	10^6-10^7	10^6-10^8	10^6-10^7	10^6-10^8	No difference between Striatum and Cortex
	Neuronal Proportions (%)				
FGF-2 Low to high	2.5-4.5	1.0-2.6	2.5-4.0	0.5-2.5	No difference between Striatum and Cortex
EGF High to low	2.5-10.0	0.1-4.5	3.5-8.5	0.0-5.5	No difference between Striatum and Cortex
	Yield (theoretical cell number)				
FGF-2 Low to high	10^2-10^4	10^3-10^5	10^6-10^8	10^7-10^9	Big difference between Striatum and Cortex
EGF High to low	10^3-10^4	10^4-10^8	10^3-10^4	10^5-10^7	No difference between Striatum and Cortex

Table 1 summarizes the results for each experiment. Each experiment was carried out separately. The data presented here represents the range across all concentrations of growth factor assessed in each experiment.

3.4 Discussion

It is reported here the effects of a range of FGF-2 and EGF concentrations on both the expansion and the numbers of neurons differentiating from ENP populations, with an ultimate view to producing donor cells for neural transplantation studies.

3.4.1 Proliferation of ENPs in response to FGF-2 and EGF

The greatest expansion of cells in this study was achieved in the presence of 10 and 20ng/ml of FGF-2 and EGF. This is supported by Minger *et al* (Minger et al., 1996) who reported optimum proliferation of mouse ENPs in the presence of 20ng/ml FGF-2. Murphy *et al* (Murphy et al., 1990) reported maximum stimulation at concentrations of 20ng/ml FGF-2 and above, and half-maximal stimulation at 1.40ng/ml FGF-2. Kitchens *et al* (Kitchens et al., 1994) also looked at various concentrations of FGF-2 ranging from 1ng/ml to 10ng/ml and reported maximum proliferation at 10ng/ml FGF-2. Ray *et al* (Ray et al., 1997) assessed the mitogenic effect of several members of the FGF family on ENP proliferation, and concluded that at a concentration of 1ng/ml FGF-2 or below, FGF-2 had a survival effect on the cells, whereas at concentrations greater than 10ng/ml, FGF-2 had a mitogenic effect with maximum proliferation observed at 20ng/ml. They also reported similar results with FGF-4. Whether concentrations greater than 20ng/ml have a more pronounced effect was not addressed in our study, although a previous study (Murphy et al., 1990) demonstrated a plateau of response above 20ng/ml.

We also looked at the effect of exposing cells to 20ng/ml FGF-2 for the first week following transfer to medium containing lower doses of FGF-2. Priming the cells in this way had no effect on cell number except when cells were transferred from 20 to

10ng/ml FGF-2. Given that there was no significant difference in proliferation for cultures expanded with 10 or 20ng/ml FGF-2 from the beginning, it is interesting that a switch from one concentration to the other after one week in culture should have such a negative effect. Whilst the decrease in concentration of FGF-2 did not have a significant effect at 1 and 5ng/ml it is of interest to note that by P6 all three culture conditions were proliferating at the same rate. This is in direct contrast to cells grown under constant growth factor conditions where cells with higher growth factor levels were expanding more rapidly than those under low growth factor levels. Therefore one could speculate, that continuing the experiment for more passages would lead to a significant difference at all concentrations, given that there was a trend for the rates of proliferation to diverge, with cells in 5ng/ml proliferating more, which was not the case in cultures that had been primed. If such was the case, then this would be in contrast to reports by Ciccolini (Ciccolini, 2001) in which it was shown that priming the cells had a positive effect on the proliferation of cultures. However, in the Ciccolini study, after priming, the cells were starved of any mitogens for several days and then re-exposed to the mitogens at the same concentration at which they were primed. The switch in concentrations may have an effect on proliferation or cell death or both and specific assays of cell death and BrdU incorporation may help to understand this. Further studies are required to understand what is happening to these cells during this time.

It was also shown in the present study that optimum proliferation of murine striatal and cortical-derived ENPs can be achieved equally well by 10 or 20ng/ml EGF, with the FGF-2 concentration held constant at 20ng/ml. Svendsen (Svendsen *et al.*, 1997) has shown previously that mouse ENPs expand for long periods of time in culture and

still maintained the ability to differentiate into neurons, astrocytes and oligodendrocytes. This is in contrast to rat ENP cultures in which the cells undergo a finite number of divisions (Kelly et al.in press). In contrast to the results presented here, Kitchens (Kitchens et al., 1994) found that in EGF-only medium there was no significant difference between 1 and 10ng/ml on the proliferation of C17-2 ENPs, which are an immortalized clonal progenitor cell line from the neonatal murine cerebellum.

In the present studies EGF and FGF-2 were not analysed alone but always in combination and therefore an understanding of the changes in responsiveness of these cultures to either growth factor can not be addressed. However, it has been shown that the emergence of EGF responsiveness with increasing gestational age is consistent with *in vivo* studies demonstrating increasing expression of EGF mRNA from E14 onwards in the developing mouse CNS (Kornblum et al., 1997; Lazar and Blum, 1992), and the observation that E14 mouse embryonic precursors can only respond to EGF after 4-5 days in culture (Reynolds and Weiss, 1992b; Svendsen et al., 1995; Reynolds and Weiss, 1992a), presumably due to up-regulation of the EGF receptor as the precursors mature. There is also evidence that EGF-responsiveness is primed by exposure to FGF-2 (Ciccolini and Svendsen, 1998), and that E8.5-derived mouse CNS precursors, which proliferate only in FGF-2, can give rise to EGF-responsive cells (Tropepe et al., 1999). Indeed, CNS precursors grown from chimeric mice homozygous for the FGF-1 receptor, which is thought to be the major receptor for FGF-2, show poor proliferation in response to FGF-2 at early gestational ages and poor response to both FGF-2 and EGF at a later age, implying that the priming with FGF-2 is essential for the development of EGF responsiveness (Tropepe et al., 1999).

3.4.2 Differentiation of ENPs cultured in FGF-2 and EGF

Looking at the neuronal proportions and neuronal yield of cells differentiated from these cultures is a step in obtaining an understanding of the characteristics of the cultures. When ENPs, grown in the various concentrations of FGF-2, were allowed to differentiate in the absence of mitogen, the greatest neuronal proportions were seen in cells from the lower FGF-2 concentrations. This is in accordance with Qian (Qian et al., 1997) who looked at the effects of FGF-2 concentrations on mouse E10 cortical ventricular zone cells. They concluded that low or basal levels (0-10ng/ml) of FGF-2 produced almost pure populations of neurons whereas higher concentrations of FGF-2 induce the generation of both neurons and glia. However, a calculation of the theoretical neuronal yield in the present study revealed that the greatest overall neuronal yields were seen in the presence of 20ng/ml FGF-2 for both striatal and cortical-derived cultures. The effect of exposing ENPs to 20ng/ml of FGF-2 for the first week, before subsequent reduction of FGF-2 level, was to increase the neuronal proportion after differentiation in culture. However, the effect on yield was much less clear-cut once adjustment was made for cell proliferation.

As with FGF-2, analysis of the neuronal proportions of striatal and cortical-derived cultures exposed to EGF concentration variations demonstrated that at the lower concentrations of EGF a greater number of neurons were observed. The proportion of neurons generated at the lower concentrations of EGF was greater than those generated at the lower concentrations of FGF-2. This is consistent with the role of EGF in astrocyte precursor proliferation and differentiation. In this study the FGF-2

concentration was maintained at 20ng/ml, thus resulting in a greater proportion of neurons.

Striatal neuronal precursors appeared to be more responsive to lower concentrations of EGF, i.e. 1ng/ml than to the higher concentrations assessed, and this was more marked than in the cortical cultures. Jori (Jori et al., 2003) found that EGF at 20ng/ml impaired neuronal differentiation in postnatal day 3 rat cultures, and resulted primarily in the production of type 1 astrocytes. Reynolds and Weiss (Reynolds and Weiss, 1996) carried out a clonal and population analysis of murine striatal ENPs and their responsiveness to EGF. They found that in response to EGF close to 90% of the primary generated spheres differentiated into the 3 principle cell types of the CNS, but with the predominant cell type being the astrocyte.

Cortical-derived cultures presented no significant difference in neuronal yield for 1, 5 and 10ng/ml, but all produced significantly higher yields of neurons than did 20ng/ml, hitherto generally perceived as the optimal EGF dose. The differences in the neuronal differentiation between the FGF-2 and EGF experiments (1a and 1b) suggest that EGF, which was always at a high concentrations in experiment 1a, was driving the proliferation and differentiation of astrocytes in these cultures, whereas in 1b where FGF-2 was maintained at a high concentration and EGF could be given at a lower concentration, there was an increased tendency for neuronal differentiation.

The neuronal yield, which takes into consideration the proliferation of cultures as well as the proportion of neurons, showed 10 and 20ng/ml to produce maximum neuronal

yields in striatal-derived cultures, with 20ng/ml being marginally better. In cortical-derived the optimum neuronal yields were from ENPs exposed to 10ng/ml EGF.

When considering the effects of FGF-2 and EGF in this study, it is important to recognize that ENPs are probably a heterogeneous population comprising neuronal progenitors, glial progenitors, and small numbers of true multipotential stem cells. One interpretation of the results in this study is that neuronal progenitors proliferate maximally at lower concentrations of FGF-2, with little increase in expansion at higher doses, and that the greater numbers of cells seen at higher FGF-2 concentrations are due to the proliferation of astroglial progenitors. Indeed, in this study, the predominant cell type at all passages and at all concentrations of FGF-2 was the astrocyte (data not shown, but see Figure 3.8).

Optimising the conditions for expansion of ENPs is of great importance if these cells are to be considered an alternative for cell replacement therapy in neurodegenerative disease. However, whilst obtaining a large quantity of cells is central to overcoming the logistical constraints associated with current supplies of human foetal tissue, it is imperative that we establish the characteristics of these cell populations. To date, most studies that analyse the effects of growth factor such as EGF and FGF-2 on ENPs focus almost entirely on the effects of these factors on proliferation. This study underlines the importance of carefully considering the effects on differentiation and is one of the first studies to do so in detail.

A further consideration is that the time window for neuronal progenitor proliferation may be limited, and this would be consistent with the gradual decline in neuronal

proportion with subsequent passages. This idea would also be consistent with the finding that neuronal proportion is increased by exposure to higher levels of FGF-2 in the first week. That is, FGF-2 may be capable of driving neuronal progenitor division soon after ENP isolation, after which the ENP populations gradually switch to astroglial production. This would be in accordance with the events of normal development, in which neuronal generation predominates until the latter part of gestation, after which astroglial production predominates. The effect of 1 week priming exposure to higher levels of FGF-2 was less pronounced in cortically-derived compared to striatally-derived ENPs, perhaps due to differences in the developmental stage of these two brain regions. FGF-2 may also have an effect on neuronal progenitor survival as well as proliferation (see chapter 4).

This synergy between EGF and FGF-2 has been described previously in E14.5 germinal zone-derived mouse precursors, where the effect was noted in high density (as used in our study), but not low density, cultures (Tropepe et al., 1999). It has also been seen in E15 rat striatal precursors (Svendsen et al., 1997a), and E13.5 mouse mesencephalic precursors (Santa-Olla and Covarrubias, 1995). However, the nature of this synergistic effect is not clear and whether or not it represents a priming effect in this context has not been resolved. Arsenijevic (Arsenijevic et al., 2001) has reported that EGF and FGF-2 have a distinct mode of action on ENPs in culture which may in part be through an intracellular mechanism. In these experiments it was reported that FGF-2 promoted the survival of the precursor state of 50% of ENPs for 6 days *in vitro* and when EGF was used in conjunction with IGF the proliferation of murine ENPs was induced by an autocrine secretion of IGF. Maric (Maric et al., 2003) has shown that FGF-2 responsive, but not EGF responsive, neural precursor

cells can sustain a calcium-dependent self-renewal and together EGF and FGF-2 permit the initial commitment of embryonic neural precursors into neuronal and glial phenotypes.

The present results emphasize a need to seek alternative methods for increasing neuronal numbers. This may require investigating a range of mitogens to stimulate neuronal progenitor proliferation, methods for improved survival of neuroblasts, and possibly inhibition of astroglial generation. However, a prerequisite will be to understand the dynamics of this system by examining neuronal progenitor turnover using agents such as BrdU (see chapter 4). Finally, it will ultimately be crucial to determine empirically the effect of FGF-2 and EGF concentration on the differentiation of ENPs in transplantation paradigms, in order to establish the relationship between neuronal differentiation *in vitro* and the survival, differentiation, and functional effects of these cells *in vivo*.

Chapter 4

BrdU analysis of the proliferation of ENPs and the quest for survival enhancing factors.

Summary

The exact role of FGF-2 and EGF in the proliferation and neuronal differentiation of ENPs is not yet known. It is clear that the presence of these mitogens has a major role in the proliferation of ENPs in culture but whether these factors act purely as proliferative agents or are as survival factors (or both) for ENPs and their differentiating progeny is unknown. Here, this question is explored by exposing ENPs that are proliferating in the presence of FGF-2 and EGF, to BrdU for a 24hr period prior to differentiation, to assess how many of the neurons differentiating from these populations were derived from actively dividing precursors. It is concluded that there is proliferation of a neuronal precursor population of cells within the cultures over at least 6 passages. Potential survival enhancing factors were also explored in differentiating ENPs and it was found that the addition of CNTF, BDNF, NGF and HGF had no effect on the neuronal numbers.

4.1 Introduction

ENPs proliferate in culture in the presence of growth factors such as FGF-2 and EGF as described in the previous chapter. However, it is not clear whether FGF-2 and EGF are acting purely as proliferating factors or also as survival factors for ENPs, given the low percentage of neurons reported from our previous experiments. Specifically, given the fact that neuronal number declines over time, the possibility existed that neurons were produced within the first passage or two only, and that the decline was then due to loss of post-mitotic neuronal progenitors that are not supported by the culture conditions. 5-Bromo-2-deoxyuridine (BrdU) is an analogue of the radioactive form of thymidine and can be used *in vitro* to analyse cell proliferation. It is incorporated into the cell's DNA thus labelling the dividing cell. By directly relating this to the neuronal differentiation of the cells one can determine whether FGF-2 and EGF are enhancing the proliferation of this population of neuronal precursors or whether their primary effect is to simply promote the survival of these cells in the culture system.

In this chapter, ENPs were expanded in culture in the presence of FGF-2 and EGF and BrdU was added to the culture for 24 hours prior to passaging and differentiation in order to assess the number of new neurons being born in culture. A second issue is addressed here, which is whether it is possible to promote the survival of neurons developing from ENP cultures by the addition of other trophic molecules such as: cilliary neurotrophic factor (CNTF), brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and hepatocyte growth factor (HGF) to the differentiating medium.

CNTF is a member of the alpha-helical cytokine superfamily. It acts through the CNTF receptor-α (CNTFRα), leukaemia inhibitory factor (LIF) receptor-β, and gp130 and is believed to initiate signalling by activating the Jak/Tyk family of kinsases (Murphy et al., 1997; Richardson, 1994). It has been shown that degenerating motor neurons are extremely sensitive to the trophic effects of CNTF and neuroprotection effects of CNTF have been demonstrated on cholinergic, dopaminergic, GABAergic and thalamocortical neurons in various lesion models and hence may have an effect in animal models of HD (for review see (Kordower et al., 2000). *In vitro* CNTF has been shown to have a protective effect on striatal neurons under excitotoxic attack by NMDA (Petersen and Brundin, 1999) and to increase the number of GABA neurons differentiating from cultures (Caldwell et al., 2001). *In vivo* studies, in which encapsulated CNTF expressing cells were transplanted to both the rat and monkey striatum prior to lesion, have demonstrated beneficial effects on both motor and cognitive behavioural tasks (Emerich et al., 1996; Emerich et al., 1997a; Emerich et al., 1997b; Emerich et al., 1998). In such experiments CNTF was shown to protect striatal neurons from degeneration induced by the toxin quinolinic acid. Phase 1 clinical trials have just been completed in which CNTF-producing BHK cells surrounded by a semi-permeable membrane, were implanted to the right lateral ventricle of 6 patients with stage 1 or 2 HD (Bloch et al., 2004; Bachoud-Levi et al., 2000). The capsule was exchanged for a new one every 6 months over a 2 year period. There were no signs of toxicity reported, although depression was observed following removal of the capsule in 3 patients. The retrieved capsules were examined and found to contain varying numbers of surviving cells with low CNTF release (Bloch et al., 2004). Overall there was no clear evidence that this technique may be useful.

NGF was first characterised by Levi-Montalcini (Levi-Montalcini and Cohen, 1956) and it was the first neurotrophic factor used in animal models of HD. NGF's mode of action is through the Trk family of receptor tyrosine kinases, TrkA, to which it binds with high affinity and the gp75 receptor to which it binds with low affinity (Schumacher et al., 1991). It has been shown that its region of influence is the cholinergic interneurons of the striatum (Gage et al., 1989; Martinez et al., 1985; Mobley et al., 1985). It is known to act on two populations of cholinergic neurons, the cholinergic projection neurons of the basal forebrain and the cholinergic interneurons of the striatum. *In vivo* it has been shown that cellular delivery of NGF to the striatum prior to lesion-induced toxicity can protect not only cholinergic neurons of the striatum but also nicotinamide adenine diphosphate reductase (NADPH-d) positive neurons (Kordower et al., 1994; Frim et al., 1993b; Venero et al., 1994; Frim et al., 1993a; Schumacher et al., 1991). This suggests that GABA-ergic neurons of the striatum can be protected from toxin induced cell death by cellular delivery of NGF. In relation to understanding the function of *htt*, it has been demonstrated in E15 rat striatal cultures, that at high concentrations of NGF there is a significant decrease in the expression of ITI5 (htt), whereas FGF-2 increased the levels in ITI5 in the same study (Haque and Isacson, 2000).

BDNF also acts through cell surface Trk receptors, in this case TrkB, and also binds to gp75 which may activate spingomyelin hydrolysis thus releasing the second messenger, ceraminde (Binder and Scharfman, 2004; Lachyankar et al., 1997). BDNF is known to enhance the neuronal differentiation of neurospheres (Ahmed et al., 1995), to promote the survival of neurons arising from the subependymal zone,

forebrain cholinergic neurons, dopaminergic neurons, and cerebellar granule neurons (Binder and Scharfman, 2004). It also mediates the activity-dependent survival of cortical neurons (Cheng and Mattson, 1994; Nakao et al., 1995). *In vitro* BDNF has been shown to inhibit free radical and apoptotic pathways and to increase the length of neurites as well as the number of branching points on the neurites and the soma area of striatal cultures (Caldwell et al., 2001; Nakao et al., 1995). *In vivo* BDNF has been shown to promote the survival and fibre innervation of striatal neurons resulting from lesion induced toxicity (Petersen et al., 2001b; Nakao et al., 1995). Recent studies in mouse models of HD have demonstrated that wild type *htt* stimulates the production of BDNF and a decrease in the cortical BDNF messenger levels correlates with disease progression thus implicating BDNF as a possible therapeutic agent for HD (Perez-Navarro et al., 1999; Petersen et al., 2001b).

HGF is a polypeptide growth factor that acts by binding to the c-Met tyrosine kinase receptor and both have been found to be expressed in the developing and mature CNS (Achim et al., 1997; Hamanoue et al., 1996; Honda et al., 1995; Jung et al., 1994). As well as its neurotrophic effects, HGF is also implicated in morphogenesis, motility, mitogenesis and antiapoptotic activities (Zarnegar and DeFrances, 1993; Isogawa et al., 2005; Akimoto et al., 2004; Gutierrez et al., 2004; Cacci et al., 2003). *In vitro* HGF has been shown to enhance the proliferation and neuronal differentiation of mouse striatal cultures (Kokuzawa et al., 2003), however little is known of its function *in vivo* in the CNS.

4.2 Experimental design:

Experiment 1

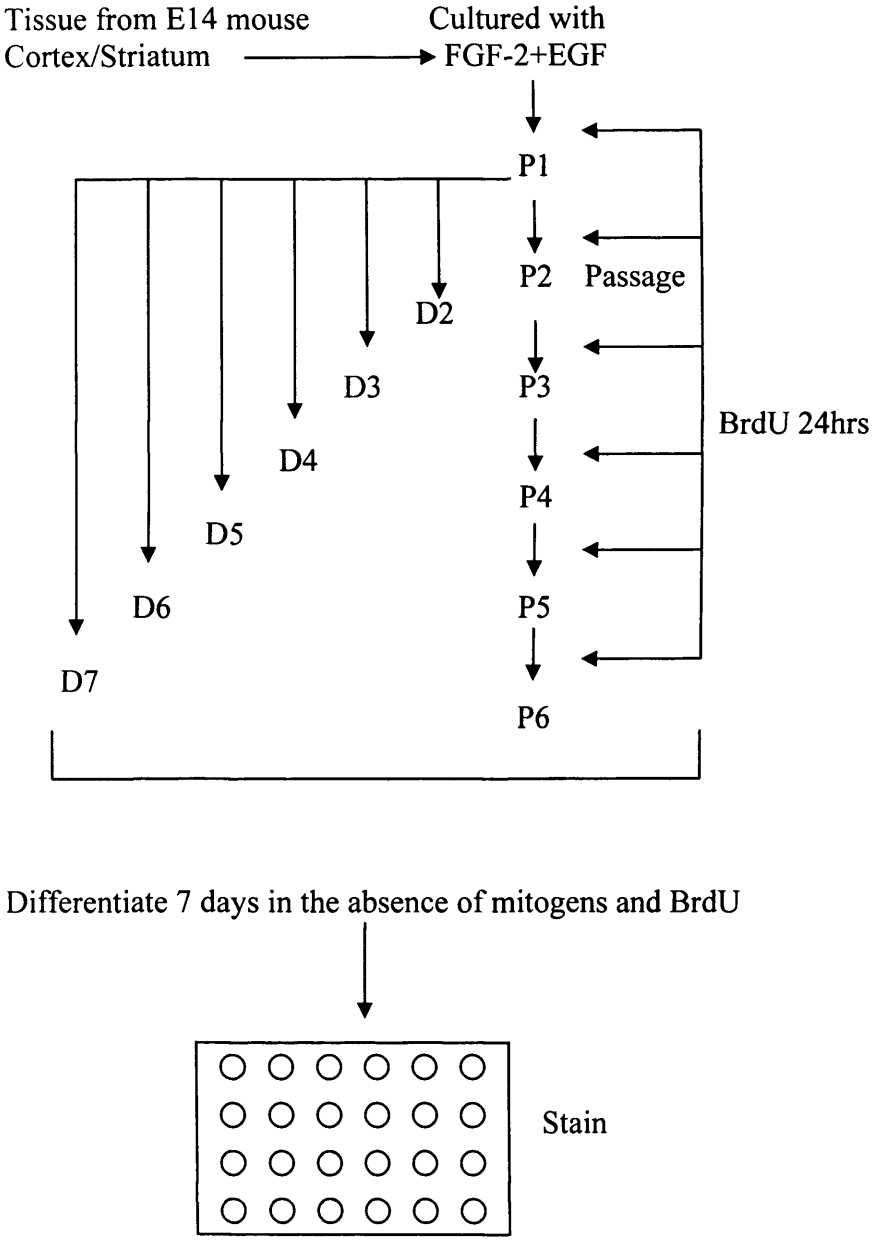


Figure 4.1. E14 mouse cortical and striatal tissue was expanded in culture in the presence of EGF and FGF-2 (both at 20ng/ml). Prior to each passage BrdU (10ng/ml) was added to the culture for 24hrs. Passaged cultures were allowed to differentiate in the absence of mitogens or BrdU and stained with antibodies for BrdU and β-III Tubulin. Between passage 1 and passage 2, BrdU was added to different populations of cells daily and after each 24hr period cells were also differentiated and stained with antibodies to BrdU and β-III Tubulin. These cells were not trypsinised before plating in contrast to passaged cultures.

Experiment 2

The rationale of this experiment was to address the possibility of enhancing the survival of differentiating neuronal precursor cells following passaging in culture. Data presented in experiment 1 showed that over time in culture, there was a decline in the proportion of neurons differentiating in these cultures, yet the percentage of BrdU positive neurons was quite high and between 2 passages (P1 and P2) the proportion of BrdU positive neurons was also high. One hypothesis was that the passaging technique of trypsinisation and trituration was having a detrimental effect on these cells and thus the effect of the addition of survival enhancing factors following passaging was addressed.

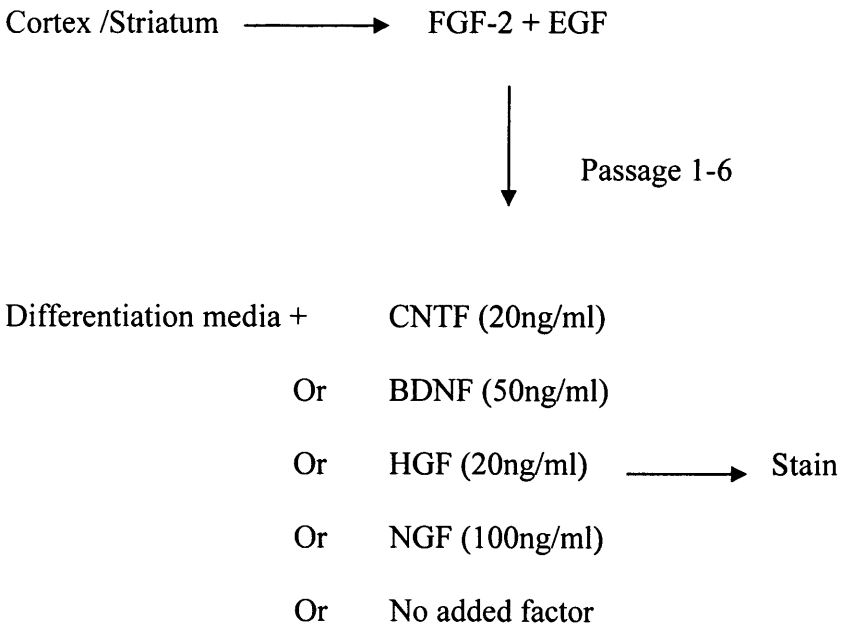


Figure 4.2. At each passage cells were plated for differentiation. Differentiation medium was supplemented with various survival factors. Control cultures had no survival factors added. After 7 days differentiation cells were stained with antibodies to β-III Tubulin, GFAP, and Hoechst and analysed for differences in overall neuronal differentiation. The concentrations of factor used were based on the most commonly used concentrations reported in the literature.

4.3 Results

4.3.1 Experiment 1-BrdU uptake over time

The number of neuronal precursors dividing in the cultures was expressed as a percentage of total neurons positive for β-III tubulin (Figure 4.3 and Figure 4.4). In the case of striatal neurons there is a highly significant effect of passage ($F_{5,12}$=28.85, p<0.001) and a less significant effect of differentiation ($F_{1,12}$=14.85, p<0.05). There was no significant difference between P1, P2 and P3, however by P4 there is a significant decline in the percentage of BrdU positive neurons (t_2=2.805, P<0.005). A similar trend was also observed in the case of cortical neurons, there was a highly significant effect of passage ($F_{5,12}$=171.68, P<0.001) and a less significant effect of differentiation ($F_{1,12}$=15.47, P<0.05). There was a significant difference at P2 to P1, P3, P4, P5, P6 (t_2=19.66, t_4=4.077, t_5=2.60, t_6=0.859, t_7=0.611, P<0.001, respectively) and by P4 there was significantly less BrdU positive neurons (t_2=2.60, P<0.001). By P5 less than 50% of all neurons are BrdU positive.

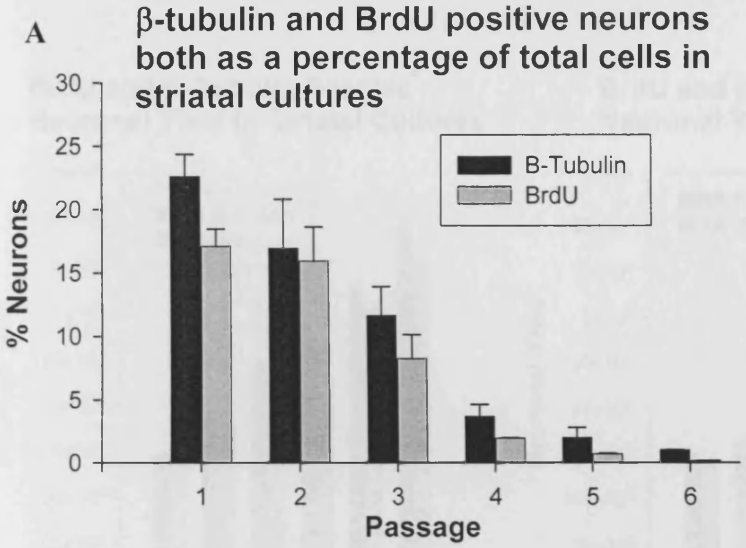
For striatal cultures there was an overall significant difference in the neuronal differentiation over time ($F_{5,12}$=171.68, P<0.001). There were no differences between P4, P5 and P6 however, P1, P2 and P3 were significantly greater than P4, P5 and P6 (t_4=2.8, p<0.005, t_5=1.32, p<0.005, t_6=0.57, p<0.005). In the case of cortical cultures there was also an overall significant difference in neuronal differentiation over time ($F_{5,12}$= 28.85, P<0.001) (Figure 4.4B). The trend was similar to that of striatal cultures in that there were differences between P1, P2, and P3 compared to P4, P5 and P6 (t_4=2.6, P<0.001, t_5=0.85, P<0.001, t_6=0.612,P<0.001). There was no difference between striatal and cortical-derived cultures.

When proliferation of the cultures was taken into account the neuronal yield increased over time (Fig 4.4) in striatal cultures only ($F_{5,12}$=3.25, $p<0.05$). There was no significant difference in the overall proliferation of striatal and cortical cultures (Figure 4.4 C).

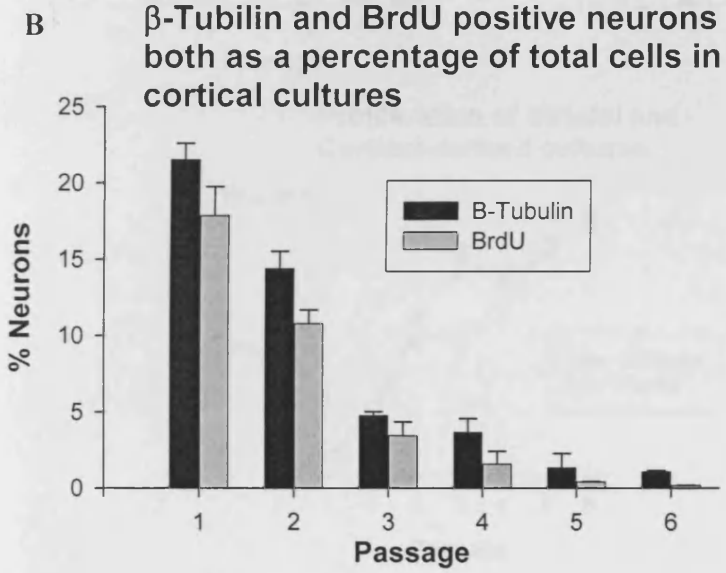
To elucidate the data further, BrdU analysis over 1 week, between 2 passages (P1 and P2) was carried out in striatal cultures only. The percentage of neurons labelling for BrdU remained high between 43% and 70% throughout. Between D2 and D4 there was a gradual decline in the percentage of BrdU positive neurons; however this was not significant on ANOVA analysis. After D4 the percentage of BrdU positive neurons increased and there was no differences at all other time points (Figure 4.6 A). When the proliferation of these cultures over the 7 days in culture (Figure 4.6C) was taken into account it was seen that there was a significant effect of day in culture on the neuronal differentiation ($F_{1,12}$=84.09, $p<0.001$) (Figure 4.6 B).

Figure 4.3. Overleaf
BrdU positive neurons are expressed as a percentage of β-Tubulin positive neurons for both striatal, A, and cortical, B, cultures. A, there was a significant difference between β-III tubulin positive neurons and BrdU positive neurons at P1 and P3. B, there is a significant difference in the proportion of BrdU positive neurons to β-III tubulin positive neurons at P1 and P2. There was an overall significant difference in neuronal differentiation over time. By P4 there was significantly less BrdU positive neurons and by P5 less that 50% of all neurons are BrdU positive.

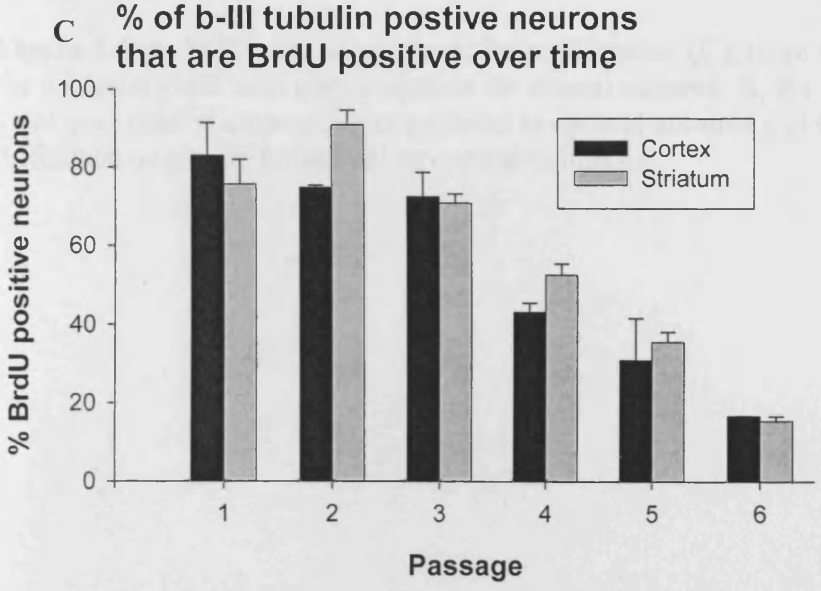
A


β-tubulin and BrdU positive neurons both as a percentage of total cells in striatal cultures
B-Tubulin
BrdU
% Neurons
Passage
0
5
10
15
20
25
30
1
2
3
4
5
6

B


β-Tubilin and BrdU positive neurons both as a percentage of total cells in cortical cultures
B-Tubulin
BrdU
% Neurons
Passage
0
5
10
15
20
25
1
2
3
4
5
6

C


% of b-III tubulin postive neurons that are BrdU positive over time
Cortex
Striatum
% BrdU positive neurons
Passage
0
20
40
60
80
100
1
2
3
4
5
6

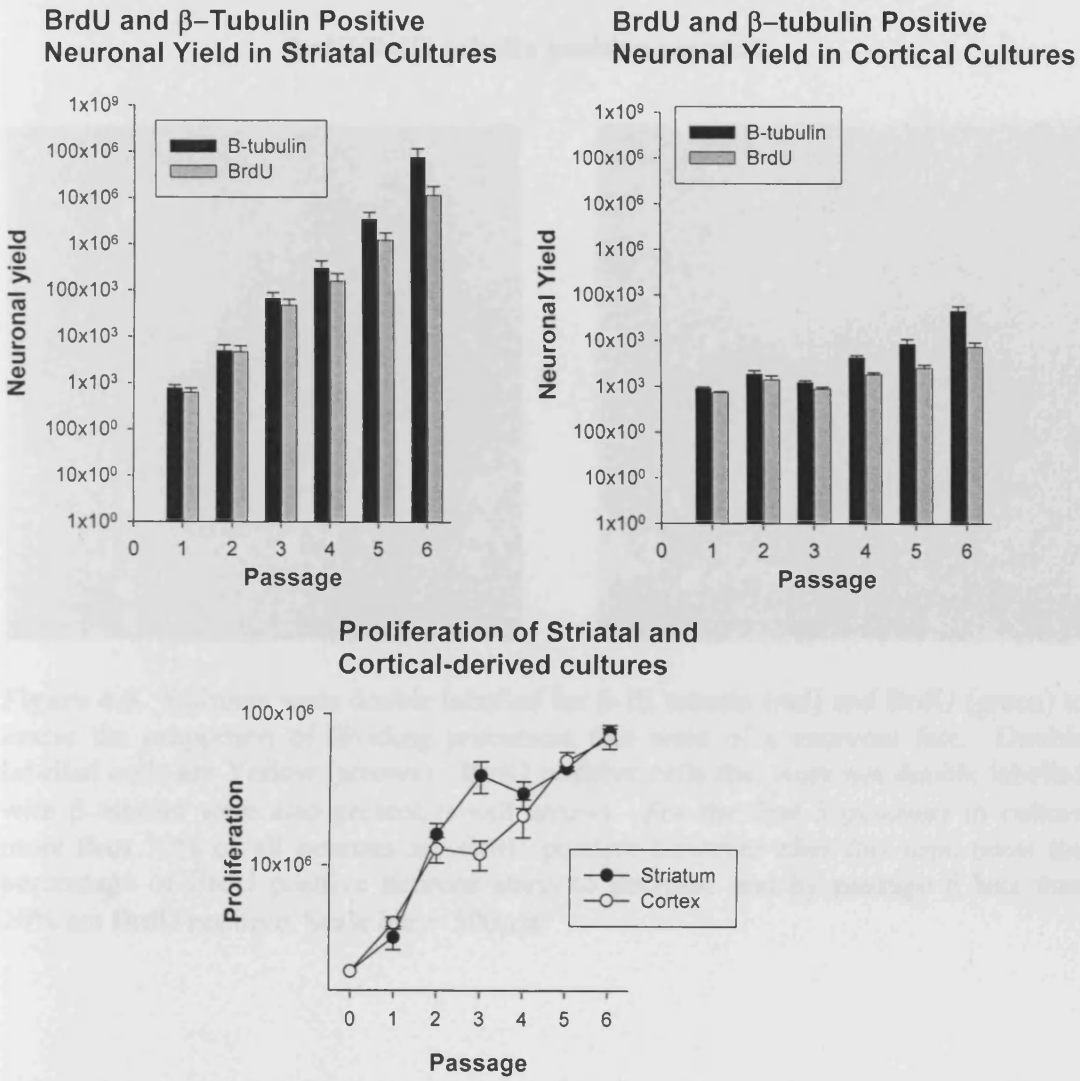


Figure 4.4 A, Following adjustments for proliferation (C), there was an increase in the neuronal yield with time in culture for striatal cultures. B, the increase in neuronal yield over time in culture is less apparent in cortical cultures and there is no major difference over time for striatal or cortical cultures.

BrdU/B-III tubulin positive neurons

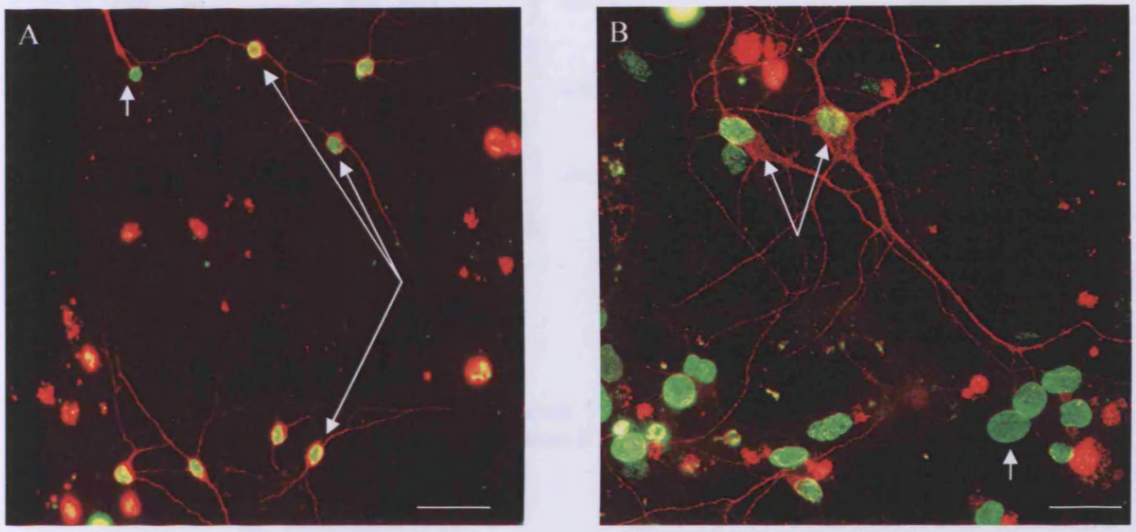


Figure 4.5. Cultures were double labelled for β-III tubulin (red) and BrdU (green) to assess the proportion of dividing precursors that were of a neuronal fate. Double labelled cells are Yellow (arrows). BrdU positive cells that were not double labelled with β-tubulin were also present (small arrow). For the first 3 passages in culture more than 70% of all neurons are BrdU positive however after this time point the percentage of BrdU positive neurons starts to decrease and by passage 6 less than 20% are BrdU positive. Scale bar = 500μm.

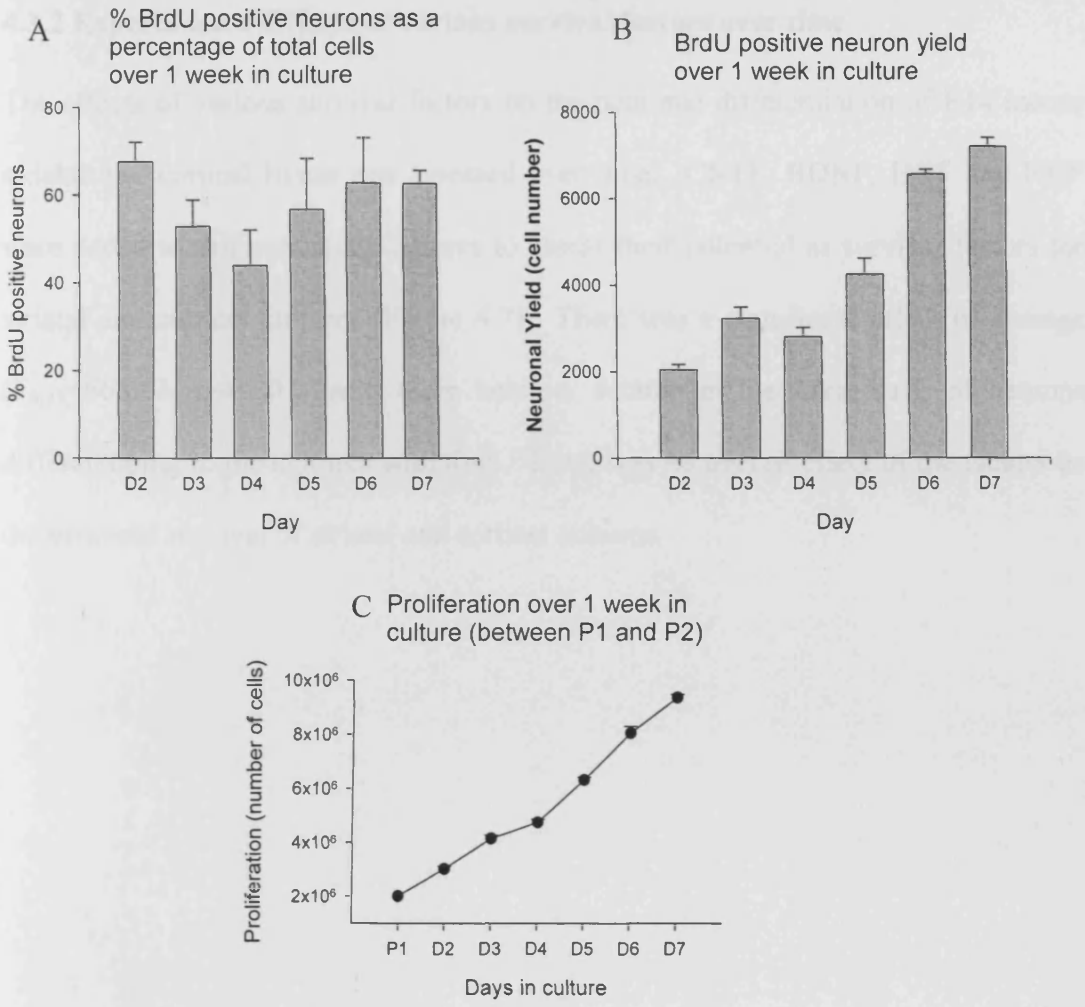


Figure 4.6. The percentage of BrdU positive neurons was quantified between two passages in culture. After passaging the percentage of BrdU positive neurons declined with there being a significant difference between D2 and D4 however after D4 this percentage increased again and there was no significant differences at all other time points.

4.3.2 Experiment 2-Effects of various survival factors over time

The effects of various survival factors on the neuronal differentiation of E14 mouse striatal and cortical tissue was assessed over time. CNTF, BDNF, HGF and NGF were added to differentiating cultures to assess their potential as survival factors for striatal and cortical cultures (Figure 4.7). There was a significant effect of passage ($F_{6,32}$=668.63, $p<0.001$) with there being a decline in the percentage of neurons differentiating in the cultures with time. There was no overall effect of the factors on the neuronal survival of striatal and cortical cultures.

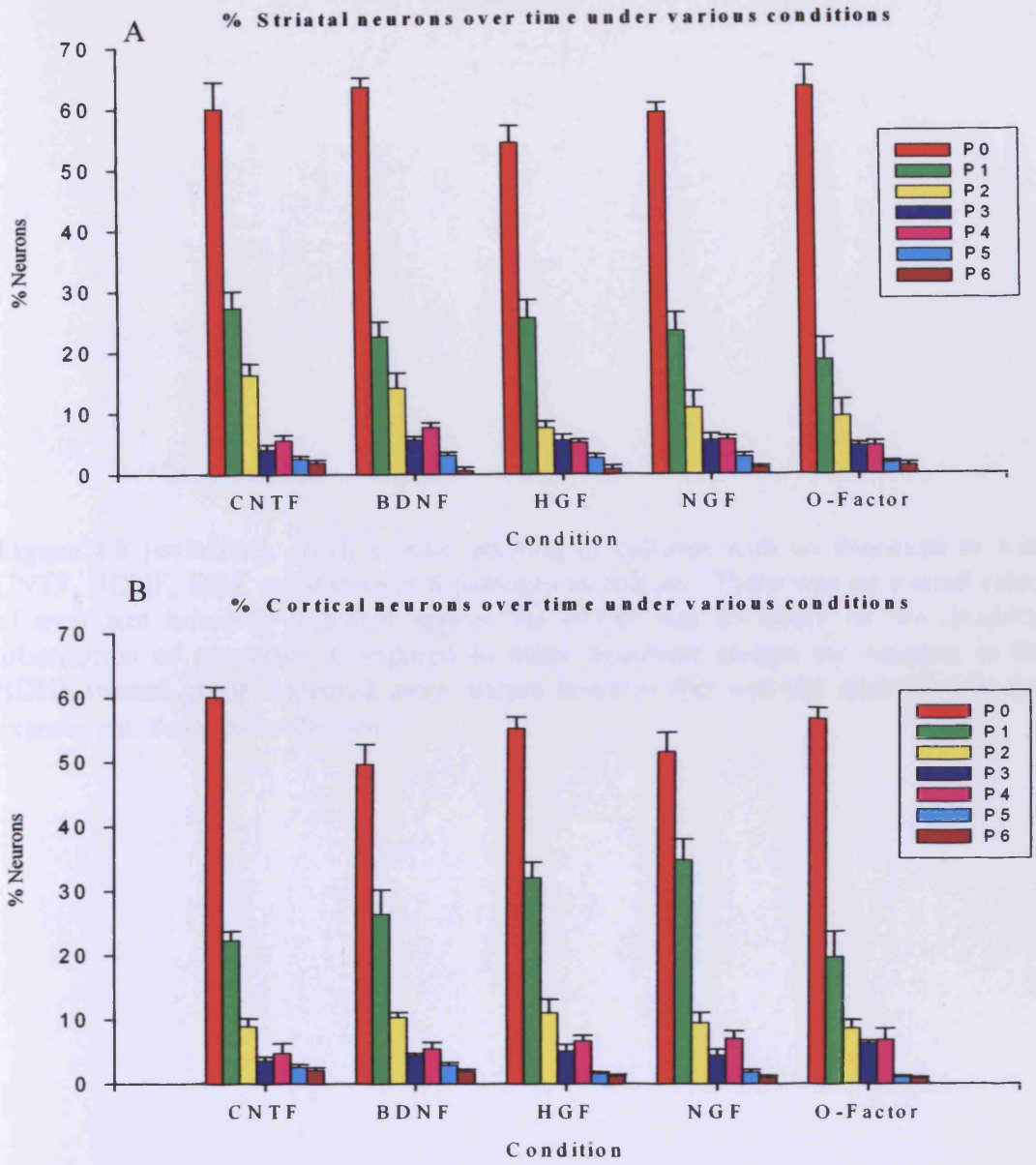
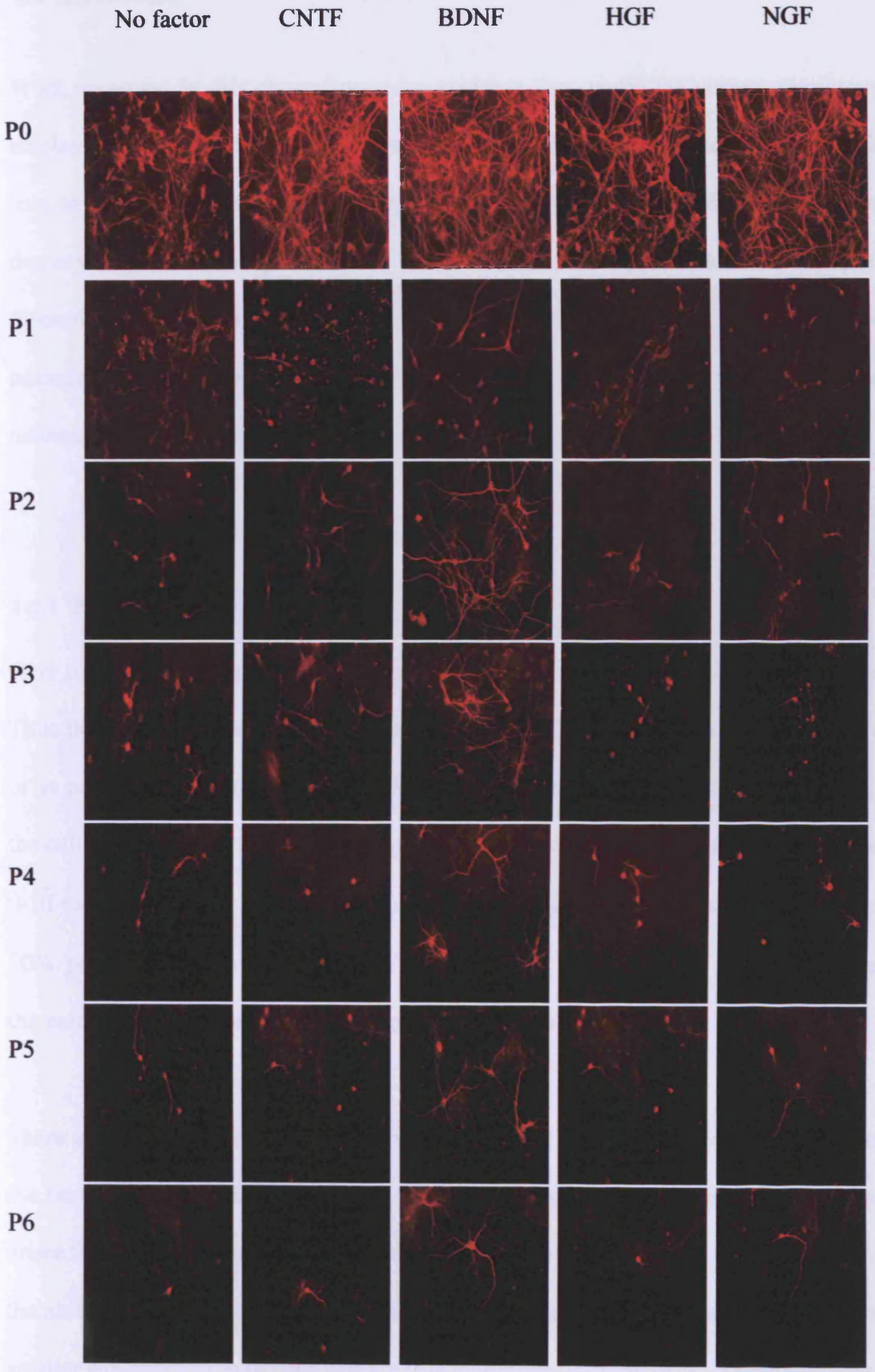


Figure 4.7. The effects of various survival factors on the neuronal differentiation of E14 mouse striatal and cortical tissue over time. A, striatal tissue, there was a significant difference at P2 between CNTF treated cultures and those treated with no survival factor and the HGF treated group. At the same time point there was also a significant difference between BDNF and HGF treated cultures. At P6 there was a significant difference between CNTF and BDNF treated cultures. B, cortical cultures, there was a significant difference at P1 between NGF and none treated cultures. There were no significant differences at all other time points.

Figure 4.8 (overleaf). β-III tubulin staining of cultures with no treatment or with CNTF, BDNF, HGF or NGF over 6 passages in culture. There was no overall effect of treatment however it would appear the BDNF has an effect on the dendritic arborisation of neurons. Compared to other treatment groups the neurons in the BDNF treated group appeared more mature however this was not quantified in this experiment. Scale bar = 200μm


No factor
CNTF
BDNF
HGF
NGF
P0
P1
P2
P3
P4
P5
P6

4.4 Discussion

Work presented in this chapter provides evidence that, under the culture conditions employed in this experiment, there is an ongoing turnover of neuronal precursors with time in culture. However, the proportion of neurons differentiating from the cultures decreased. When the proliferation of the cultures was taken into account, the theoretical neuronal yield was seen to increase with time in culture. The addition at passage of the survival factors CNTF, BDNF, HGF and NGF had no effect on the neuronal survival of either striatal or cortical cultures after passaging.

4.4.1 BrdU uptake over time

Over time in culture the neuronal differentiation from proliferating cultures decreases. Thus the question arises as to whether FGF-2 and EGF are acting as survival factors or as proliferating factors in these cultures. To understand this, BrdU was added to the cultures for 24hours prior to passaging and allowed to differentiate. BrdU positive β-III tubulin positive neurons were found at all passages. Up to passage 3 more than 70% percent of neurons were BrdU positive with there being a gradual decline thereafter and by P6 less than 20% of all neurons were BrdU positive.

There are several possibilities that may explain what is happening to these cultures; the first possibility is that most of the labelling that is observed in the early passages arises from the precursor cell population and that with time in culture these cells loose the ability to continue proliferating and are phased out of the culture system leaving a smaller sub-population of cells that are true multipotential stem cells. These true stem cells may have a slower turnover than the more restricted precursor cells or it may be

that the culture conditions that supported the proliferation of the ENPs initially no longer supports the proliferation of this population of cells. A second possibility is that the neuronal precursor cells are being diluted in the culture system by other cell types such as astrocytes. Looking at the theoretical neuronal yield from the striatal cultures, it would appear that there is a trend towards the β-III tubulin positive cells and the BrdU positive cells to diverge away from each other which would further support this possibility of other cell populations taking over the culture. A third possibility is that the neural precursor cells are subjected to extreme stress during the passaging technique and as a result are killed off. This was the hypothesis which lead to Exp 2, looking at the effects of various survival factors on the neuronal differentiation of these cultures over time. Given that there was no effect of any of the factors analysed in this study would suggest that this may not be the case. However, it is necessary to repeat this experiment adding the survival enhancing factors to the proliferation rather than the differentiation medium as it is possible that the cells require these factors before being passaged. Ultimately, however, resolving these alternatives will remain extremely difficult until we have a better understanding of the precise lineages underlying the development of neural precursors, along with selective and specific markers for each stem/precursor cell in that lineage. These are not currently available but their search is an active topic of our lab as in many others.

Figure 4.9

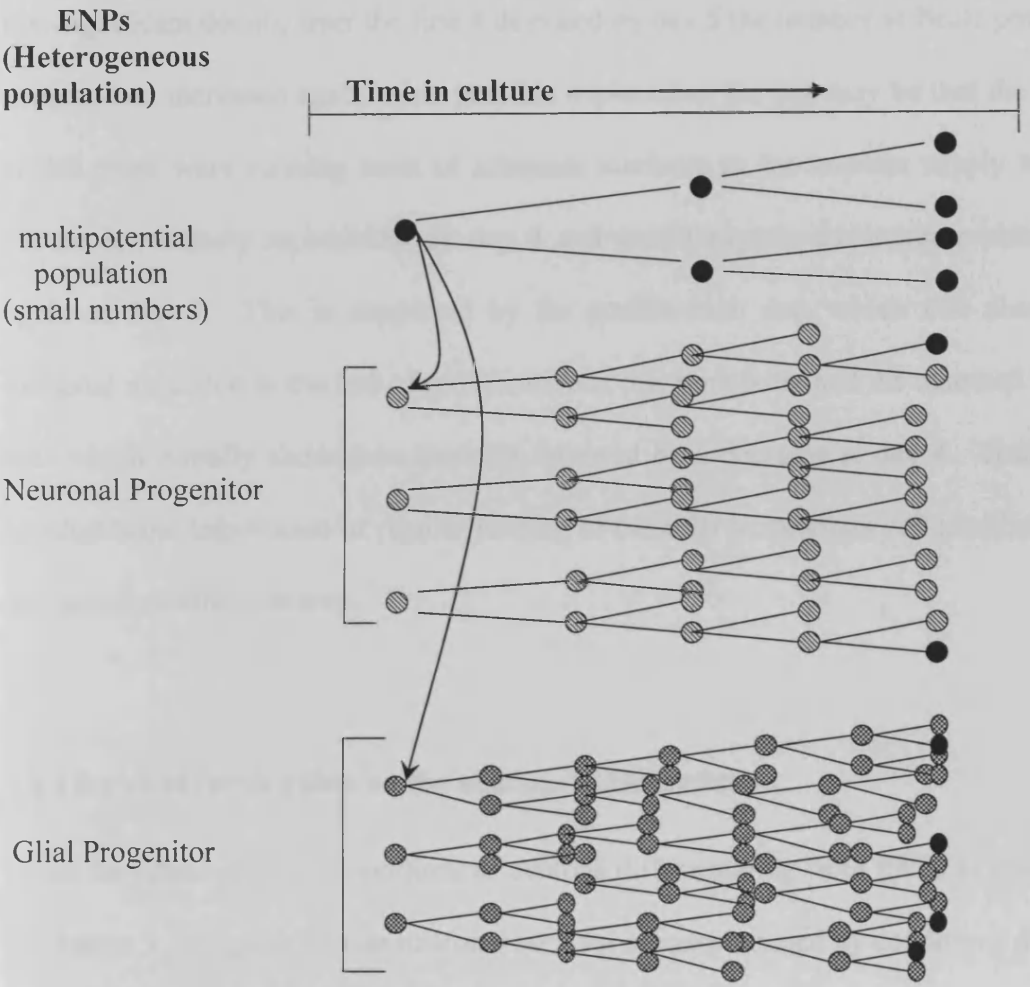


Figure 4.9 Simplistic schemata of the theoretical proliferation dynamics, it is important to emphasise that this is theoretical as the precise lineages of ENP populations, are still not fully defined.
A working hypothesis is that the multipotential stem cell within the ENP population results in low numbers of cells as their turnover is slow. The neuronal progenitor has a higher rate of proliferation than that of the multipotential stem cell and a lower rate than the glial progenitor population of cells. There is the possibility that the neuronal precursors are depleted with time or they undergo a switch at some point during their proliferation to the production of glia. Furthermore, cells that have undergone their final division and are ready to differentiate into neurons (black dots) fail to do so as they die due to unfavourable conditions.

To try and analyse the situation further, BrdU was added each day between passage 1 and passage 2 and it was found that there were no significant changes in the percentage of BrdU positive neurons between the 2 passages. However, there was a non-significant decline over the first 4 days and by day 5 the number of BrdU positive neurons had increased again. One possible explanation for this may be that the cells at this point were running short of adequate nutrients as the nutrient supply to the culture is routinely replenished on day 4 and would explain the increase observed again at day 5. This is supported by the proliferation data which also shows a marginal reduction in the rate of proliferation at this time point and the neuronal yield data which initially showed an increase followed by a decrease at day 4. Thus this highlights the importance of regular feeding of the cells to maintain cell proliferation and neuronal differentiation.

4.4.2 Survival factor effect on the neuronal differentiation.

Given the relatively low proportions of neurons differentiating from ENPs as reported in Chapter 3, it is possible that neuronal survival is compromised by conditions during passaging or differentiation. For example passaging using enzymes and trituration may have been traumatic to the cells. With this in mind Exp 2 (described above) was undertaken in which the growth factors CNTF, BDNF, HGF and NGF were added to the differentiation media of the cultures, as they have previously been reported to have effects on striatal cultures (Gregg and Weiss, 2005; Kokuzawa et al., 2003; Kordower et al., 2000; Lachyankar et al., 1997). However it was found that the survival factors chosen had no overall significant effect on the neuronal survival of these cultures. Growth factors were not combined in this study nor were the factors added to the proliferation medium. Several other potential survival factors are

available but for the purpose of this experiment those most associated with HD were chosen to be assessed.

Previously CNTF has been shown to have a survival effect on striatal cultures against NMDA toxicity but no effect was seen on calcium ionophore A23187-induced toxicity in striatal cultures, which indicates that the growth factor does not promote survival by enhancing general defences against raised intracellular levels of calcium (Petersen and Brundin, 1999). Several *in vivo* studies have shown the survival effects of CNTF in animal models of HD (Emerich et al., 1996; Emerich et al., 1997a; Emerich et al., 1997b; Emerich et al., 1998; Emerich, 1999; Emerich and Winn, 2004; McBride et al., 2004). It may be that CNTF is required during the proliferation phase or in combination with other factors to optimise neuronal survival.

In accordance with other findings (Nakao et al., 1995; Petersen et al., 2001b) BDNF appeared to have an effect on the neuritic outgrowth of neurons as seen in Figure 4.8. BDNF treated neurons appeared to have longer neurites compared to all other conditions, although this data was not quantified. In relation to HD, BDNF has been found to be a critical factor associated with the motor symptoms and disease progression (Canals et al., 2004). In mouse models of the disease cortical BDNF levels of expression have been shown to correlate with disease progression and cortical BDNF mRNAs displayed a positive correlation between cortical neuronal activation and expression of BDNF mRNA and thus expression of BDNF in cortical areas projecting to striatum is dependent on both target integrity and neuronal activity therefore having an implication in disease progression as cell death progresses (Petersen et al., 2001a; Rite et al., 2005; Zuccato et al., 2005; Canals et al., 2004).

In culture NGF has been shown to maintain the proliferation of ENPs and on removal of the mitogen, the cells were seen to differentiate implying a role in maintaining ENPs in a proliferative state (Cattaneo and McKay, 1990). NGF expression has been shown to decrease the expression of IT15 in striatal cultures (Haque and Isacson, 2000) which implicates NGF as a possible candidate for therapeutic intervention in HD. *In vivo* studies have shown NGF to have a survival enhancing effect on excitotoxically lesioned striata (Frim et al., 1993b; Frim et al., 1993a; Kordower et al., 2000; Schumacher et al., 1991; Venero et al., 1994). In this study, NGF failed to have an effect on the neuronal differentiation of striatal and cortical cultures.

In the case of HGF it has previously been shown that the addition of this factor to the differentiation conditions of EGF and FGF-2 expanded striatal cultures results in a significant increase in the percent of neurons generated (Kokuzawa et al., 2003), such an effect was not observed in this study. Little is known about the effect of this factor on striatal cultures and further work is warranted to validate its potential as a candidate neurotrophic factor.

Further studies are required to understand the dynamics of ENP cultures. One possible experiment would involve labelling the cells with the marker, succinimidyl ester of carboxyfluorescein diacetate (CFSE) which is a reliable fluorescent marker for the analysis of cell generation over time. Each cell generation can be identified by the level of intensity of the marker. Such cell populations could be isolated using a FACS sorter and differentiated in culture. Such a study would help to characterise in detail the turnover of specific cell populations within the culture. A second approach would be to add survival enhancing factors to the proliferation media of the cultures.

As well as the addition of various factors to the medium of these cultures other manipulations to improve neuroblast survival may be warranted such as culturing the cells in lowered oxygen levels as previously reported by Studer (Studer et al., 2000). Analysing cell death within these cultures also warrants investigation and this might be carried out using the CFSE method previously mentioned or again using BrdU over different time points in culture. The work presented here has been carried out in mouse ENPs and for manipulation into the clinical context it will be imperative that the same experiments are repeated with human ENPs. Clearly further work is warranted on the basis of the results presented here to fully understand the true potential of ENPs.

4.5 Conclusion

From this study it can be concluded that the expansion of ENPs in the presence of FGF-2 and EGF supports the proliferation of neuronal precursors for a period of time in culture, whether this is a long lasting effect is not clear from this study and further experiments are required to answer this question. However, it would appear that the addition of survival factors to the differentiation condition has no effect on the survival of neurons. Thus, much work is required to understand fully the dynamics of this system and to determine ways of maintaining a significantly high proportion of neurons of a specific phenotype.

Chapter 5

Characterisation of primary and 10 day expanded human ENPs in a rat model of HD.

Summary

The potential of human derived ENPs to differentiate into striatal like DARPP-32 positive neurons both *in vitro* and *in vivo* was assessed in this study and compared to that of primary foetal striatal tissue. It was found here that after 10 days expansion in culture ENPs maintained a striatal phenotype although the number of neurons differentiating from the culture decreased. *In vivo* striatal positive markers were also observed, albeit at a much lower level which may be a result of the relatively short time these grafts were given to differentiate, 12 weeks. However, it is promising that 10 days expansion in culture can yield a population of cells that maintain a striatal like phenotype that can also be seen *in vivo*.

5.1 Introduction

ENPs from the developing striatum will proliferate, and if expanded for short periods of time *in vitro* differentiate to form DARPP-32 positive neurons, a surrogate marker for the GABAergic, medium sized spiny projection neurons. The expression of DARPP-32 has been shown to correlate with the degree of functional recovery on many tests (Fricker et al., 1997b; Nakao et al., 1996; Nakao et al., 1999; Watts et al., 2000b). In the previous chapter it was shown that there was a reduction in the capacity of ENPs for neuronal differentiation over time in culture. This is confirmed by transplant studies which show a reduction in the neuronal differentiation and graft survival of long term expanded ENPs (Armstrong and Svendsen, 2000; Zietlow et al., 2005). Thus, more work is needed to determine whether long term expansion of ENPs have a clinical potential. However, despite the modest increase in cell number of short term expanded ENPs, the possibility that these cells retain the capacity to differentiate into medium spiny neurons, may represent a real and useful clinical opportunity. Therefore the aim here is to characterise these cells with a view to considering them as a potential donor source for striatal repair.

In relation to the current situation where primary foetal tissue is used as the tissue source for transplantation there are huge pragmatic constraints on the therapy, and hence the importance of such investigations looking into the potential of ENPs as an alternative tissue source. Although modest, the increase in cell number over a 10 day period in culture could have a substantial influence on clinical studies at this stage of the process where no other alternatives are available. If the 10 day expansion of these cells was sufficient for the generation of an increased number of functional medium

spiny projection neurons over the number obtainable from the starting striatal material, it may then pave the way for further clinical studies in neural stem cells.

Work presented in this chapter aimed to characterise the potential of hENPs compared to primary foetal tissue. Specifically the potential of 10 day expanded ENPs to survive transplantation in a quinolinic acid lesioned striatum and to maintain a striatal phenotype both *in vitro* and *in vivo*.

5.2 Experimental Procedures

5.2.1 Experimental Design

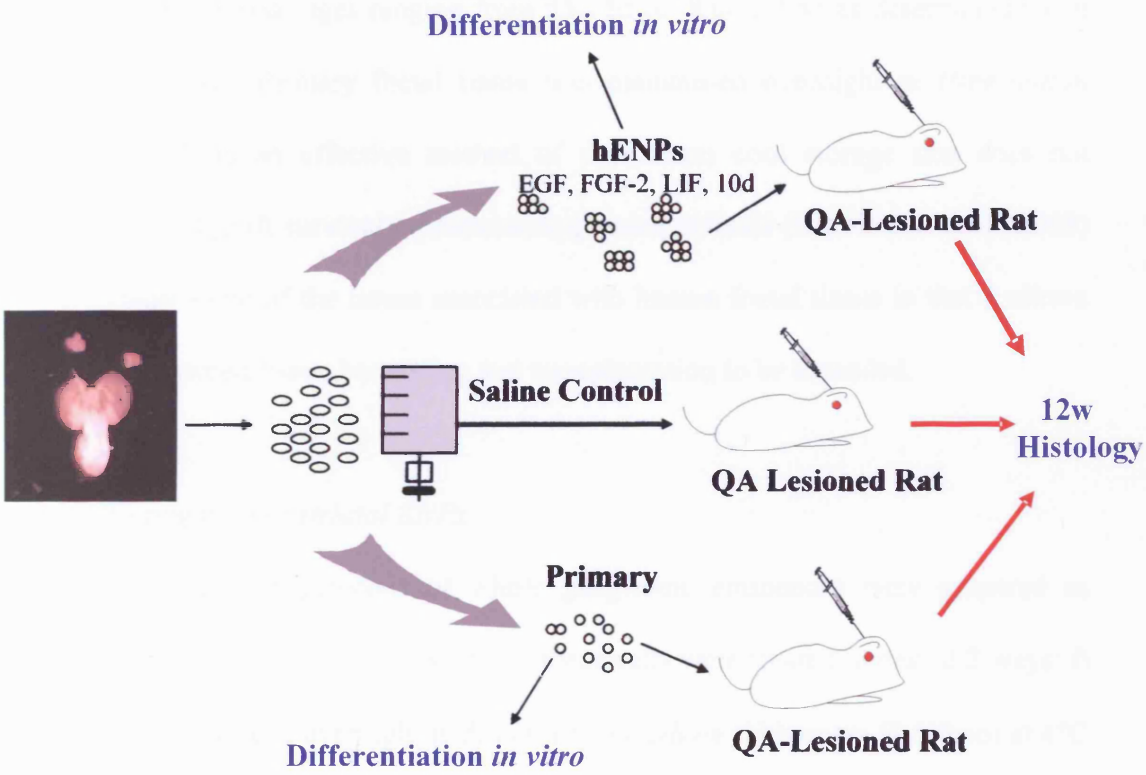


Figure 5.1 Schemata of the experimental design. There were 14 animals in each of the graft groups and 8 control animals, all animals received a 45nmol quinolinic acid (QA) lesion 1-3 weeks prior to grafting and all animals were immunosuppressed with cyclosporine A (CsA) for the duration of the experiment starting one day prior to grafting.

5.2.2 In vitro experiments

5.2.2.1 Human Tissue

Whole ganglionic eminences from three human foetuses were used in these studies, with post conceptional ages ranging from 55-75d (c. 8.0-10.5w) as determined by *in utero* ultrasound. Primary foetal tissue was maintained overnight in *Hibernation medium* which is an effective method of short term cool storage that does not adversely affect graft survival in immunosuppressed animals (Hurelbrink et al., 2003) and alleviates some of the issues associated with human foetal tissue in that it allows an interval between tissue harvesting and transplantation to be extended.

5.2.2.2 Propagation of striatal ENPs

Coarse single cell suspensions of whole ganglionic eminences were prepared as described in Chapter 2. For *in vivo* study, these cells were treated in one of 2 ways: ***i***) Stored as primary cells overnight in *Hibernation medium* (Hibernate E, Gibco) at 4°C at a density of 500,000 cells/ml; or ***ii***) Expanded as ENPs for 10 days (Chapter 2). Briefly, 200,000 cells/ml were seeded in B27 *proliferation medium* supplemented with EGF (20ng/ml), FGF-2 (20ng/ml) heparin (5μg/ml) and LIF (10ng/ml). ENPs were fed, by replacing half the medium with fresh medium containing twice the concentration of B27, EGF, FGF-2 and LIF, every 4 days.

5.2.2.3 Characteristics of ENPs in vitro

The characteristics of striatal ENPs following 10 days propagation were assessed *in vitro* as described in Chapter 2. Briefly, spheres were dissociated to a coarse single cell suspension and plated onto poly-L-lysine-coated coverslips at a density of $10x10^4$ cells in 30μl differentiation medium. After 4-6 hours cells were flooded with 500μl

of differentiation medium and allowed to differentiate for 7 days prior to fixation. Cells were fed by replacing half the medium with fresh medium every 3 days.

Indirect fluorescent immunocytochemistry was performed using standard protocols (Chapter 2) with primary antibodies directed against β-III Tubulin (1:1000), DARPP-32 (1:30,000) and GFAP (1:1000). Fluorescent staining was visualised on a Leitz DRMB microscope, and cell counts performed at x40 magnification. Pseudocolour fluorescent images were obtained using Openlab 2.1 image analysis software.

5.2.2.4 Neural Transplantation

All animals were administered Cyclosporine A (CsA) daily starting one day prior to transplantation. The primary graft group (n=14) received intrastriatal grafts of primary human striatal tissue, hibernated over night. Following 10 days expansion in culture the second group of animals (n=14) received an intrastriatal graft of cells. All cell implants were performed as described in Chapter 2 and consisted of 2μl injections of 500,000 cell suspensions delivered over 2 mins. Briefly, for ENP grafts, an aliquot of the cells was dissociated, via a trypsin digest and mechanical trituration, to single cells, allowing an estimate of the cell number and viability within the sphere suspension as well as allowing cells to be differentiated for *in vitro* characterisation. Using this information, undissociated spheres were harvested by centrifugation and resuspended in DNase at 250,000 viable cells/μl. Hibernated tissue was prepared for transplantation by washing the tissue three times in DMEM/F12, determining the viable cell count and resuspending the cells in DNase at a density of 250,000 viable cells/μl.

5.2.2.5 Histology and immunohistochemistry

Twelve weeks following transplantation animals were transcardially perfused and their brains processed for histological analysis. Serial coronal 40μm frozen sections were prepared, collected and stored as described in Chapter 2. A 1:12 series of sections were processed for Nissl staining using haemotoxylin and eosin (H&E) and histochemically stained for AChE A further 1:6 series was processed for indirect single label immunohistochemistry with the following primary antibodies:

Mouse anti-HuNu (1:1500) (human specific nuclei)

DARPP-32 (1:30,000)

Calbindin (1:20,000)

Parvalbumin (1:4,000)

The basic protocol was identical in all cases and is described in Chapter 2. Visualisation was via the DAB method. Staining controls consisted of omission of the primary antibody and these confirmed the specificity of staining in all cases.

5.2.2.6 *Quantification of graft parameters*

Graft volume was determined as described (Chapter 2) on a 1:12 series of Nissl-stained sections.

5.3 Results

5.3.1 In vitro characteristics

Striatal ENPs used for transplantation underwent a 1.94x increase in absolute cell number after 10 days, when characteristic spheres were seen in suspension. Tissue maintained in hibernation medium for up to 24 hours maintained viability above 92% in all cases based on trypan blue exclusion assay analysis.

Primary and expanded tissue was differentiated for 7 days *in vitro* prior to fixation and histological analysis. Cells were stained for DARPP-32 and β-III tubulin (Figure 5.2). Primary tissue consisted of an average of 72% neurons, 75% of which were DARPP-32 positive neurons (55% of all cells were DARPP-32 positive). After 10 days expansion there was a reduction in the total number of neurons differentiating in culture from 72% to 43%, 64% of which were DARPP-32 positive neurons (28% of all cells were DARPP-32 positive) (Figure 5.3 A and B). There is a significant difference in the neuronal differentiation between primary and 10 day expanded cultures (t_2= 43.479, $P<0.05$) with there being more neurons in the primary than the 10 day expanded cultures, and a similar significant difference in the DARPP-32 neuronal differentiation (t_2= 29.344, $P<0.05$).

Primary and 10 day expanded human striatal tissue positive for DARPP-32

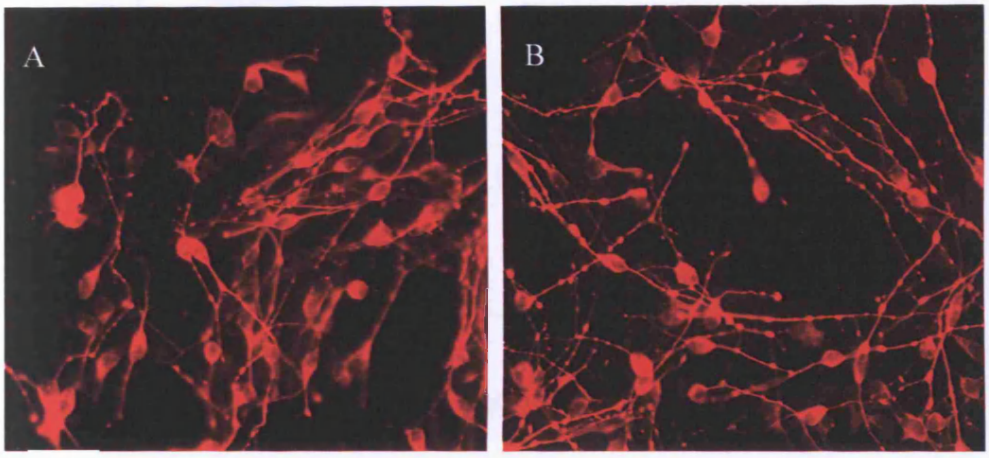


Figure 5.2. Fixed cultures were immunohistochemically stained for DARPP-32 and β-III tubulin. DARPP-32 immunopositive staining of A) Primary and B) 10 day expanded hENPs. There was no significant difference in the proportions of neurons staining for DARPP-32 between primary and 10day expanded cultures.

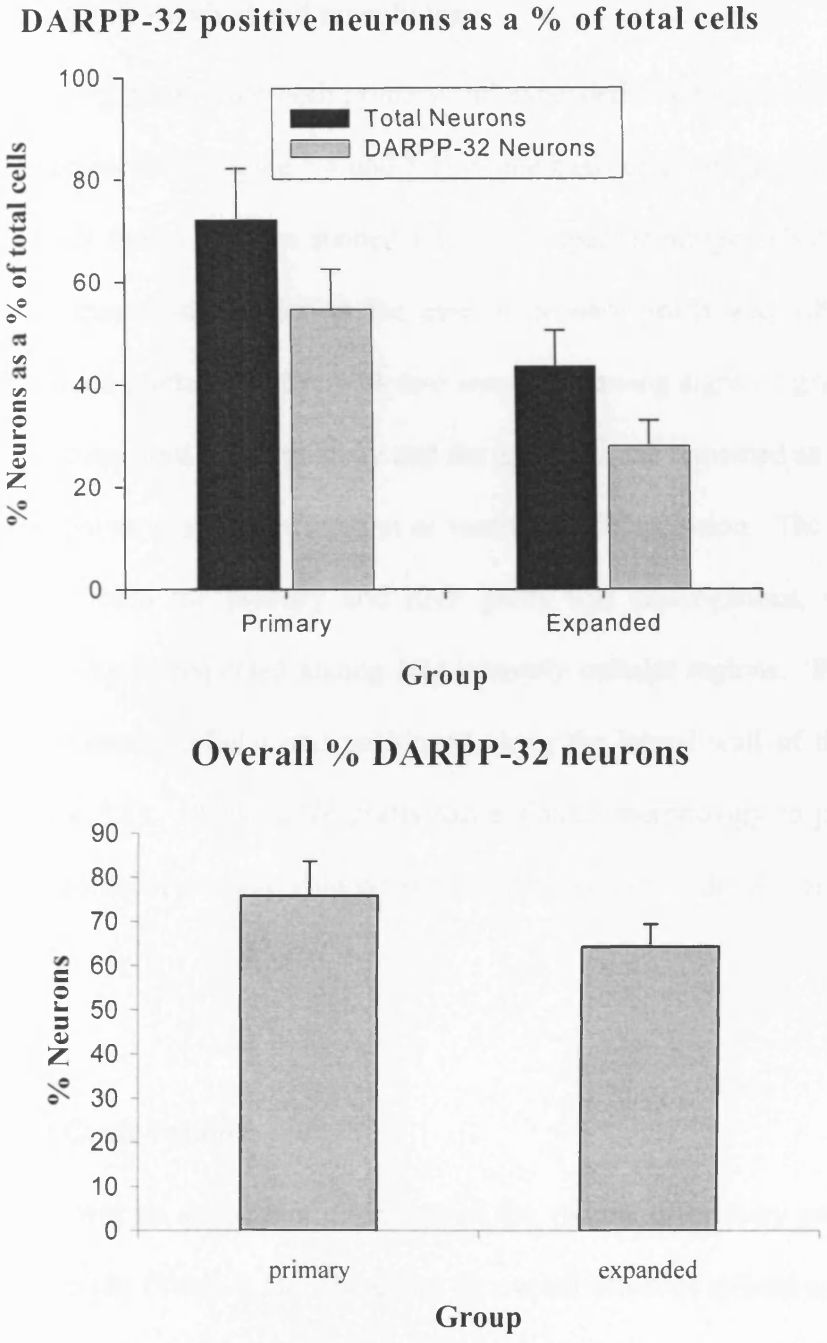


Figure 5.3 A) Total neuronal differentiation was based on β-III tubulin immunoreactivity as a percent of total cells as was DARPP-32 neuronal differentiation. There is a significant difference in the neuronal differentiation of both β-III tubulin and DARPP-32 positive neurons between primary and 10 day expanded cultures and B) The percent of total neurons that were DARPP-32 positive for primary and expanded cultures. There is no significant difference between the primary and expanded groups.

5.3.2 Graft survival and morphology

Surviving grafts from both primary and expanded tissue could be identified in Nissl-stained sections (Figure 5.4 and 5.5) on the basis of cytological and cytoarchitectonic features and in sections stained for human specific antigen (HuNu). Graft survival with immunosuppression in the case of primary grafts was 100% and for 10 day expanded grafts was 86% with two animals showing signs of graft rejection. Grafts were consistent in morphology and the grafted tissue remained as a distinct mass with no apparent vascular infiltration or ventricular compression. The cellular distribution within, both the primary and ENP grafts was heterogenous, with zones of high cellularity interspersed among less intensely cellular regions. Primary tissue grafts were densely cellular and positioned along the lateral wall of the caudate-putamen (Figure 5.4). 10 day ENP grafts had a similar morphology to primary tissue grafts with zones of densely cellular regions interspersed with less dense regions (Figure 5.5).

5.3.3 Graft Volume

There was no significant difference in the volume of primary and 10 day expanded ENP grafts (Table 5.1). There was an overall effect of striatal and ventricle volume resulting from the lesioned induced striatal atrophy, for all groups ($F_{1,26}$=289.47, P<0.001, $F_{1,26}$=166.95, P<0.001 and $F_{1,26}$=189.57, P,0.001 respectively). There was a significant difference in the volume of the lesioned striatum and the non-lesioned striatum for primary and 10 day expanded grafted animals (t_2=21.49, P<0.001) with the non-lesioned striatum being significantly larger than the lesioned. As a result of the lesion there was also a significant difference in the ventricular volume with the ventricle on the lesioned side significantly larger than that on the non-lesioned side

(t_2=4.32, P<0.001). Control animals also presented with a decrease in striatal volume and an increase in ventricular volume arising from the lesion (t_2=12.93, P<0.001 and t_2=8.24, P<0.001 respectively). Calculating control volumes allowed for a measure of graft compensation to be made and it was found that neither primary nor 10 day expanded grafts significantly compensated for the striatal atrophy associated with the lesion.

Primary graft in the lesioned host.

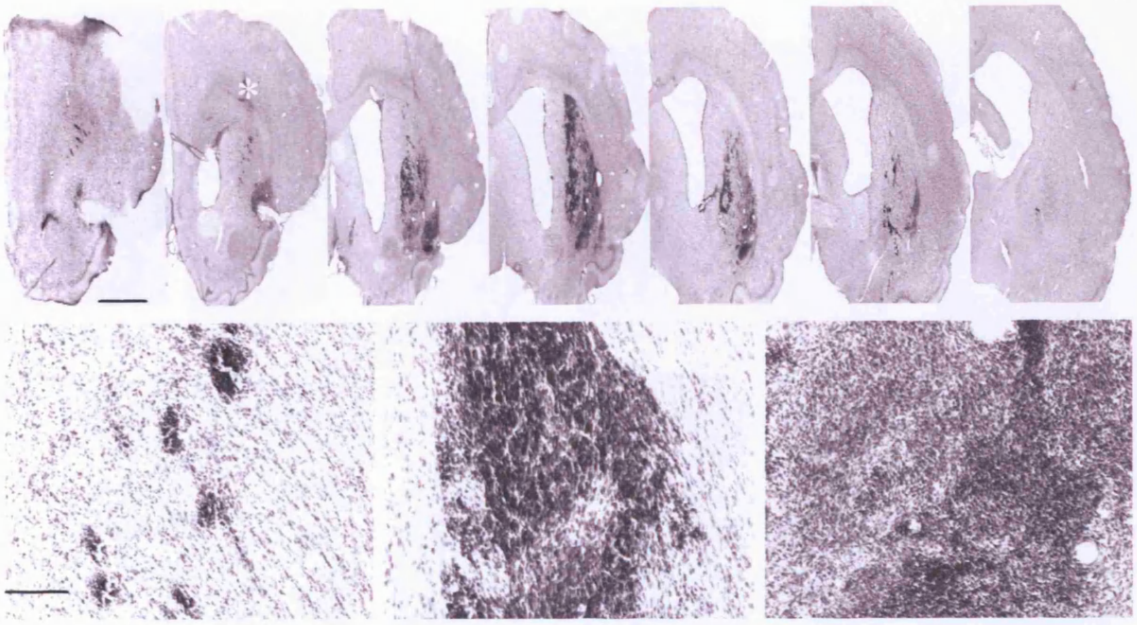


Figure 5.4 Graft morphology of a primary graft as demonstrated by Nissl staining. Distributed neuronal profiles are present throughout the graft with zones of densely cellular areas interspersed with less dense areas (higher power images, lower panel). Zones of necrotic cavitation are evident and some reflux up the implantation tract is also evident (indicated by *). Scale bar = 500μm

Expanded graft in the lesioned host.

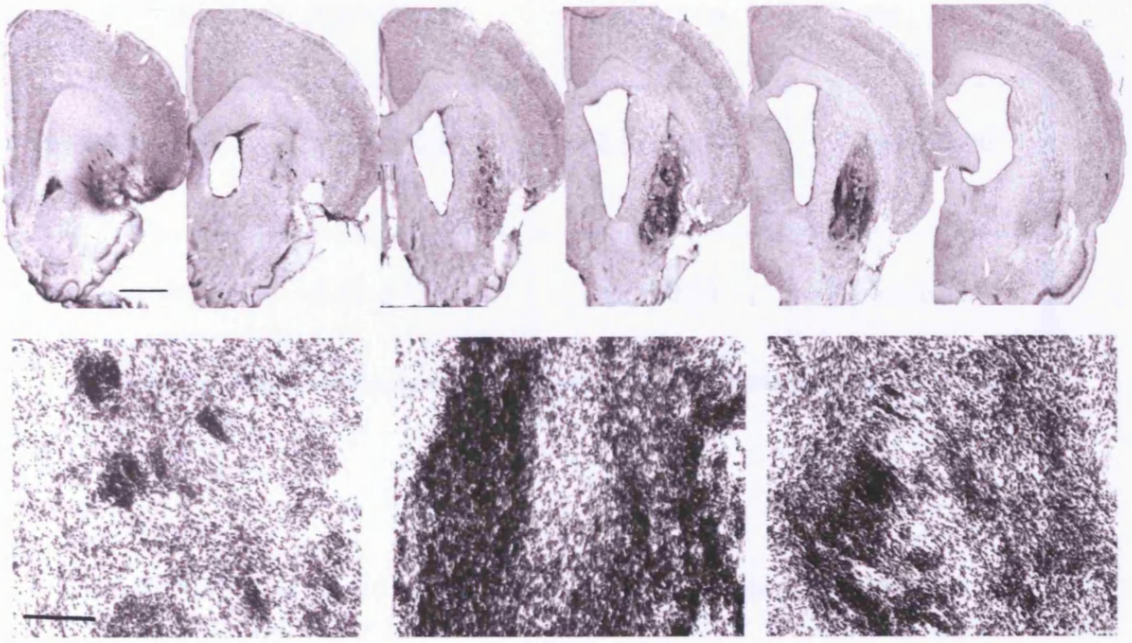


Figure 5.5 The graft morphology of an expanded graft is similar to that of primary grafts as demonstrated by Nissl staining. Distributed neuronal profiles are present throughout the graft with zones of densely cellular areas interspersed with less dense areas (higher power images, lower panel). Scale bar = 500μm

	Primary	Expanded	Control
Graft Survival	100%	86%	*na*
Graft Volume	3.59 ± 0.47	4.34 ± 0.56	*na*
Striatal Volume	13.86 ± 0.85	12.12 ± 0.98	13.65 ± 0.96
Ventricle Volume	8.42 ± 1.0	8.03 ± 0.53	8.46 ± 0.81

Table 5.1 There is no significant difference in the graft volume of primary and 10 day expanded hENP grafts after 12 weeks *in vivo*. As a result of the lesion induced striatal atrophy there is an overall significant difference in the striatal volume of the lesioned and non-lesioned side for both primary and 10 day expanded grafts as well as for control animals. Likewise, there was an overall significant increase in ventricle volume for all groups.

5.3.4 Expression of striatal markers in vivo

Grafted animals were analysed for the expression of the striatal markers DARPP-32, calbindin, parvalbumin, and AChE and expression of all markers was relatively sparse. Control animals that received a sham graft to the lesioned striatum displayed a typical near complete loss of DARPP-32 expressing neurons on the lesioned side and associated striatal atrophy.

DARPP-32 positive cells were evident in graft regions and were seen to double label with HuNu (Figure 5.6). Between subjects there was heterogeneity in the number and the distribution of DARPP-32 positive neurons and in general immunoreactivity was low. However, numbers of DARPP-32 neurons were counted and although there appeared to be more DARPP-32 positive neurons in the 10 day expanded graft group compared to primary grafted animals, this failed to reach statistical significance (Figure. 5.7). DARPP-32 positive neurons within both the primary and expanded grafts adopted an immature morphology when compared to those on the non-lesioned side and there was some heterogeneity in the morphology of DARPP-32 positive cells between the graft groups, however on analysis this was not significant. In all subjects analysed there was no evidence of DARPP-32 positive projections across the graft-host boundary (Figure. 5.8 A, D, G and J). AChE activity was diffuse throughout the body of the grafts and there were some P-zones in primary grafts but this stain was weak and there were few clear P-zones of dense AChE activity in 10 day expanded grafts (Figure 5.9).

For parvalbumin stained sections, no immunopositive cells were identified within the grafts, however immunopositive fibres were seen in the graft. Sections were double

labelled for HuNu to identify that immunopositive cells were graft derived (Figure 5.8 B, E, H, and K). Low levels of cabindin positive cells were identified within the graft area of both primary and 10 day expanded and grafts but not all calbindin positive cells were HuNu positive (Figure. 5.8 C, F, I and L).

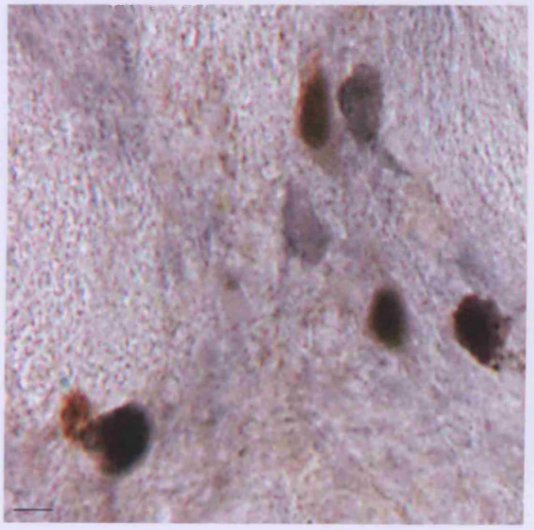


Figure 5.6 HuNu/DARPP-32 positive cells within the graft. Scale bar = 500μm

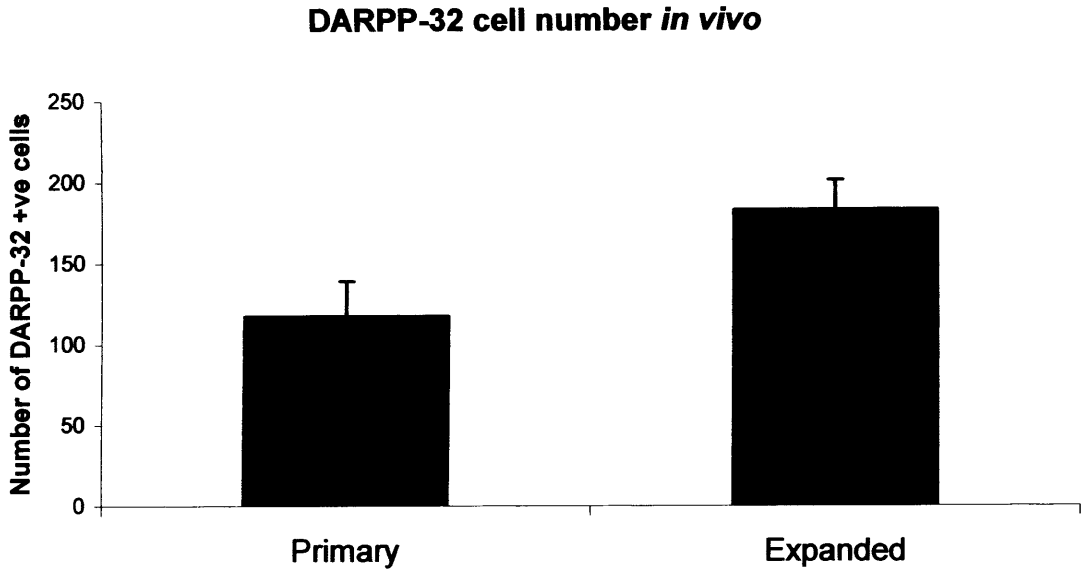


Figure 5.7. The number of DARPP-32/NeuN positive cells within the graft area. There was no significant difference between primary and 10 day expanded grafts.

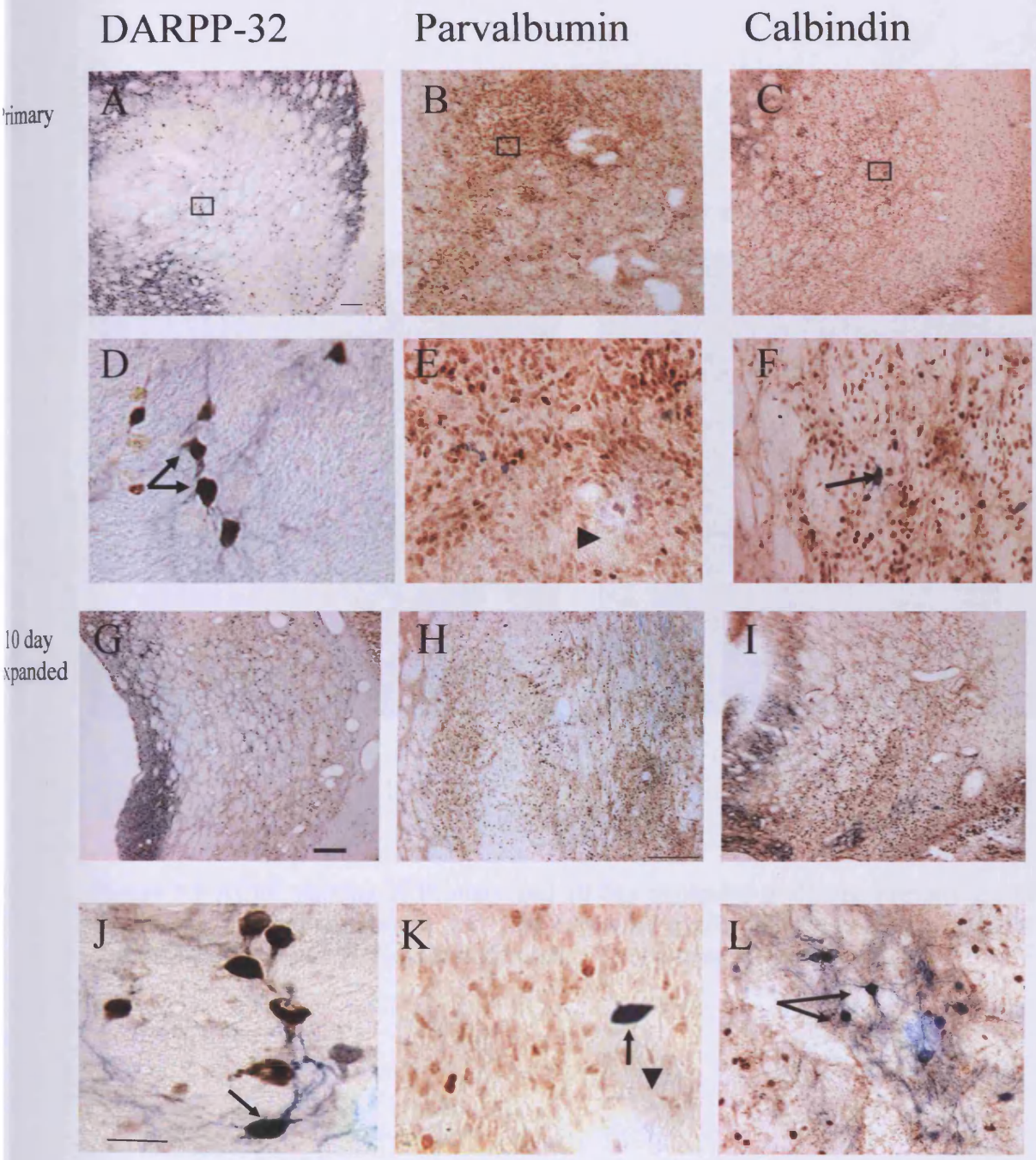


Figure 5.8. A,D,G and J, DARPP-32/NeuN positive cells in Primary (A and D) and expanded (G and J) grafts. B, E, H and K, Parvalbumin positive cells were only occasionally seen within the graft but none were seen to double label with HuNu (arrow in E and K). Parvalbumin positive fibres were seen within the graft area (arrowhead in E and K). C, F, H and L, Calbindin positive /HuNu positive cells within the graft (arrows in F and L). Scale bar = 500μm

Primary **10 day expanded**

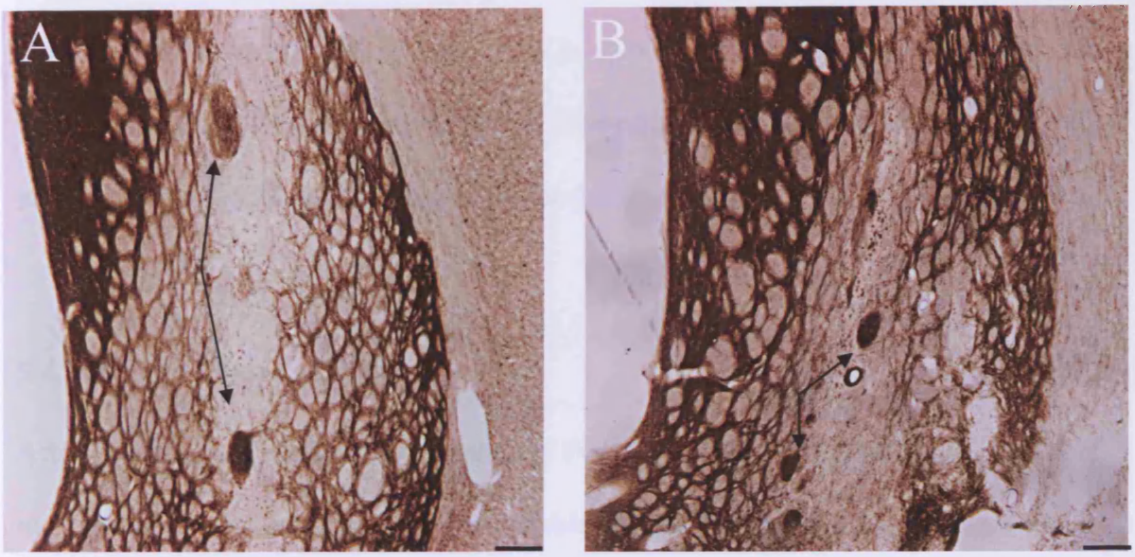


Figure 5.9 AChE staining of Primary and 10 day expanded grafts the primary grafts show weak P-zones (arrows in A). The expanded grafts also show P-zone activity (arrows in B) although perhaps less frequent P-zones were seen in this study.

5.4 Discussion

Work presented in this chapter supports the notion that hENPs have the potential to maintain a striatal phenotype for at least 10 days expansion in culture. 10 day expanded tissue *in vitro* retains a strong striatal phenotype as seen with the high proportion of DARPP-32 positive neurons present after this short expansion time. However, the true potential of these cells *in vivo* is difficult to understand given the low striatal specific marker expression in both primary and expanded grafts and long term survival of grafts will be important for this.

5.4.1 In vitro nature of ENPs

After 10 days expansion in the presence of FGF-2, EGF and LIF there was a 1.94 fold increase in the total number of cells available for grafting which suggests that there is an initial phase of cell death that is then followed by a proliferative phase. Several studies have reported specific differentiation of such cells in disease models after similar expansion times (Armstrong et al., 2000; Armstrong et al., 2003b; Minger et al., 1996; Studer et al., 1998; Svendsen et al., 1997a; Zietlow et al., 2005). Populations of hENPs appear to change with time in culture and with passaging in relation to their phenotypic differentiation potential and this will need further analysis for an understanding of the clinical potential of long term expanded cells. In culture there is a relatively small increase in cell number, 2x after 10 days expansion. Whilst the proportion of DARPP-32 neurons differentiating from the cultures decreases, there is a 1.7x increase in DARPP-32 neuronal yield. Therefore, longer expansion times would result in more cells or alternatively other methods that would increase the expansion of these cultures could be employed (see below).

Chapter 3 addressed the issue of mitogen concentration; however, there are other factors that have been identified that may be crucial for the optimal culture of these cells such as lowered oxygen levels and the free floating rollo tube expansion of these cells. Studer (Studer et al., 1996) reported a novel method for preoperatively testing the levels of dopamine in culture systems using a free floating rollo-tube expansion culture system. This method allowed the selection of the optimum dopamine secreting cultures for transplantation in models of PD. In another study by Studer (Studer et al., 2000) the oxygen levels at which cultures were expanded was reduced from 20% to 3±2% and an increase in the proliferation of VM-derived cultures was reported and also a 9 fold increase in the dopaminergic neuron differentiation of the cultures. Erythropoietin was also shown in the same study to increase the neuronal differentiation of these cultures. These or similar strategies could be used to increase the yield of short term expanded striatal cells.

5.4.2 Graft survival

Twelve weeks following transplantation, all primary grafts survived and almost all (12/14) ENP grafts survived, thus suggesting that the inflammatory environment of the acute excitotoxic striatal lesion is compatible with the survival of these cells (Duan et al., 1998). There was no significant difference in survival or graft volume between primary and 10 day expanded graft groups. In the quinolinic acid lesioned striatum there is a decrease in the striatal volume with a resulting increase in the ventricle size as a result of the cell death of striatal neurons. In this study, there was no compensation in striatal or ventricular volume in the graft groups compared to control animals.

5.4.3 Graft phenotype of primary and 10 day expanded ENPs

Nissl staining of both primary and 10 day expanded grafts showed that the grafts consisted of zones of densely cellular areas that were interspersed with less dense areas. The donor origin of these cells was also confirmed by immunohistochemical staining for human nuclear antigen (HuNu).

Characteristic striatal markers such as DARPP-32, AChE, calbindin and parvalbumin were examined in this study. All grafts displayed a diffuse AChE activity and DARPP-32 immunopositive neurons were few and morphologically immature. There was strong evidence of a P/NP zone organisation within both primary and expanded grafts. DARPP-32 medium sized spiny neurons are heterogenous in nature and are composed of neurons that co-express substance P and the D1 dopamine receptor and those that express met-enkephalin and the D2 dopamine receptor. In normal development those that co-express substance P and the D1 dopamine receptor would be involved in the direct pathway whereas those expressing met-enkephalin and the D2 dopamine receptor would be involved in the indirect pathway (Campbell et al., 1995b; Graybiel et al., 1989a; Campbell et al., 1995a). The P-zones of striatal grafts also contain interneurons and glia (Graybiel et al., 1989b; Graybiel et al., 1989a) whose function in striatal graft function is uncertain. Thus it is evident that if ENPs are to be a true alternative for neural transplantation in HD they should be able to yield a range of striatal phenotypes *in vivo*.

It is known that in rat to rat isografts DARPP-32 positive neurons are first seen 4-5 days following transplantation and by 6 weeks there are rich P zones of DARPP-32 neurons and AChE activity (Labandeira-Garcia et al., 1991). Sirinathsinghji

(Sirinathsinghji et al., 1993) has also shown that such grafts are fully mature by 12 weeks post transplantation by examining the expression of growth associated protein (GAP,43), a gene associated with immature neurons. In the case of human to rat xenografts this period of maturation appears to be considerably extended (Grasbon-Frodl et al., 1996; Naimi et al., 1996; Pundt et al., 1996a; Pundt et al., 1996c). One such study in which human fœtal tissue of a similar gestational age as that used in the present study, found weak DARPP-32 immunoreactivity as well as diffuse AChE activity and a paucity of P zones after 17 weeks *in vivo* (Pundt et al., 1996c) . Several studies in which xenografts derived from species with extended gestations such as human (Armstrong et al., 2000; Brundin et al., 1986; Englund et al., 2002b; Geny et al., 1994) and pig (Armstrong et al., 2003b; Armstrong et al., 2002; Galpern et al., 1996; Garcia et al., 1995; Huffaker et al., 1989; Larsson et al., 2000b) have been carried out and support the prolonged maturational period of xenograft tissue. Furthermore, ENPs containing a heterogenous population of cells and a large proportion of immature precursor cells and thus the maturational time line may be even more prolonged. Therefore, it seems likely that the failure of the grafts in this study to attain maturity would explain the low immunoreactivity of the striatal markers examined in both primary and expanded cells.

Although DARPP-32 immunoreactivity was low in general, it was encouraging to find immunopositive neurons in 10 day expanded grafts which suggest that after this short period of time in culture hENPs are capable of maintaining the phenotype of medium-sized spiny neurons. Calbindin reactivity was also low but present within the grafts of both primary and 10 day expanded tissue. Parvalbumin positive cells within the graft were not graft derived as none of the few positive cells identified double

labelled with HuNu. Despite this, parvalbumin positive fibres were seen traversing the graft parenchyma, which are possibly afferent fibres coming into the graft.

Understanding the true potential of ENPs in this study has been restricted by the low neuronal maturation and subsequently the reduced expression of neurochemical phenotypes. The use of daily immunosuppression hinders long term studies from being carried out as the animals become unwell and die and thus for further more long term studies to be carried out a model system is required that will overcome the need for immunosuppression (see Chapter 7). Another possible explanation for the delayed maturation may be that extrinsic epigentic signals are required for phenotypic induction and are lacking in the transplant environment and this is supported by the demonstration that exposure of FGF-2 expanded rat mesencephalic precursors to serum factors prior to grafting may increase phenotypic differentiation (Studer et al., 1998).

5.5 Conclusions

In this chapter it has been shown that hENPs have the potential to maintain a striatal phenotype both *in vitro* and after grafting after a relatively short period (10 days) of expansion in culture. However, full characterisation was hindered by overall low differentiation within the grafts. Further studies with extended post grafting survival times are required to better characterise the differentiation potential of these cells. Other experiments are required to look at extended expansion times of the cells *in vitro* as well as alternative methods for the improved expansion of ENPs in culture. Work toward this is addressed in chapter 7.

Chapter 6

Graft neuronal projections in the host environment.

Summary

Reconstruction of the circuitry lost as a result of the disease state is a major aim of neural transplantation. Animal studies have shown that primary foetal transplants have the ability to send neuronal projections from the graft to host target areas such as the globus pallidus and substantia nigra. Whether ENPs share this potential or even if they can send projections more extensively in the brain is not fully understood. It has been shown that human ENPs can project widely in the adult host and there are suggestions that ENPs are better than primary cells in this respect although the specificity of these projections is unknown (Armstrong et al., 2000). In such studies projections could be an intrinsic feature of ENPs or they could be a product of the xenograft environment. This chapter compares both primary and expanded foetal grafts in the allo- and xenograft environment. The xenograft environment may be permissive to long projections from the donor tissue as the donor tissue may not recognize the stop signals present in this environment. Several labelling techniques were employed to validate the outgrowth of the graft including; GFP transgenic mice, LacZ labeled cells and iontophoretic injection of the anterograde tracers BDA, neurobiotin and PHA-L. Neuronal projections were seen from all grafts although ENP grafts sent out projections more extensively. When comparisons were made between mouse and human grafts it was observed that human ENPs had a greater potential for long distance fibre outgrowth than mouse ENPs.

6.1 Introduction

One of the assumed requirements of neural transplantation is that the grafted cells be able to reform the circuitry lost as a result of disease. Thus, it is important that the grafted cells can communicate with the host and it is supposed that the closer the reconstruction to normal, the better. For full reconstruction it may be important that a graft can project to distant sites. In the case of Huntington's disease, the grafted cells are required to reform the lost links previously formed by the medium spiny neurons. In allografts of primary neural tissue it appears that donor projections cross the graft host boundary, but it is not clear that they travel long distances (For review see (Wictorin, 1992). Armstrong (Armstrong et al., 2000) showed that expanded human ENPs, when grafted to the lesioned rat brain, projected to distant sites. These projections were seen to cross the graft/host border as well as crossing the corpus callosum and entering the globus pallidus bilaterally. Clearly there are a number of explanations for these findings which need to be explored further. Firstly, the projections could be a property of the ENPs, that is, ENPs may be intrinsically more able to send out long projections than are primary cells. Alternatively, they may be a result of the xenograft environment i.e., the donor tissue may not recognize the 'stop' signals present in the host brain that would otherwise prevent allografted tissue from sending out long projections. Thirdly, it may be that such projections are also present in the allograft situation but have not been reported previously due to the problems of being able to reliably identify donor allograft projections.

Tracing the projections of grafted tissue is dependent on the availability of reliable markers. In the case of human to rat experiments NF_{70} reliably labels human donor

neurons and their projections but not the rat host cells. However, when grafting mouse tissue to the rat brain it becomes more complicated as there are few reliable mouse markers available. M2 and M6 are reported to label mouse neuronal and astrocytic tissue respectively; however in our hands the specificity of these two markers was markedly less reliable than generally reported, making their use for such experimental analysis limited. As a result alternative methods of labelling the grafted tissue had to be identified.

The first experimental approach was to use a transgenic mouse expressing the green fluorescent protein under a prion promoter as the donor tissue. Whilst this was the most obvious alternative to labelling the grafted cells it failed to give optimal results (see results) for the purpose required, and therefore other methods of identifying the donor cells were also explored.

Labelling of tissue for transplantation with a lentiviral (LV) vector is one alternative that has been taken in addressing this issue. An equine LV vector expressing the lacZ construct was used to infect the cells prior to grafting. LV vectors are derived from a group of highly pathogenic retroviruses, which includes the human immunodeficiency virus HIV. With properties common to the oncoretroviral vectors which are widely used, LV viruses also have the advantage of being able to infect both dividing and non-dividing cells. Their properties are favorable for long term expression of the transgenes in the nervous system in that they have a large cloning capacity, at least 9kb, and are stably integrated into the genome of the target cells (for review see (Bjorklund et al., 2000). There are safety issues regarding the use of viral vectors for clinical application, particularly those which are HIV derived and especially the

possibility of reversion to replication competent forms (Castro et al., 2001). LV vectors do not carry the same risk, as they can be attenuated to obtain replication-defective, non-pathogenic vectors.

Another approach is the use of anterograde tracers, and was also incorporated to this study using the method of iontophoresis for the delivery of these tracers to the graft site. Tracers used include; Neurobiotin, PHA-L and BDA. Neurobiotin is an amino derivative of biotin that can be used as a label for even the finest axonal arborisations and is non-toxic. In comparison to other tracers such as biocytin, neurobiotin is more soluble and it iontophoreses better and so it remains in the cells longer. It has a high affinity for avidin, which provides the basis for its detection (Luo et al., 2001; Novikov, 2001; Xue et al., 2004). *Phaseolus vulgaris* leucoagglutinin (PHA-L) is a plant lectin that has four 'L' subunits and has a high affinity to specific sugars (α-D-Manatose and β-D-galactose). It binds to glycoconjugates on the neuronal membrane and is internalized and transported along the neurites of the neuron. Its rate of transport is approximately 4.6mm/day with a survival time of 18-20 days. It can be detected with a highly specific antibody to the lectin (Wouterlood and Groenewegen, 1991; Gerfen and Sawchenko, 1984; Dolleman-Van der Weel MJ et al., 1994; Wouterlood and Jorritsma-Byham, 1993). Biotynilated dextran amine (BDA) is a high molecular weight, water-soluble dextran conjugate. BDA is transported rapidly into the neuron and along the neurites and its labelling of cellular processes is highly detailed. BDA however does not survive over long periods of time and hence is better suited to short term experiments (Reiner et al., 2000; Veenman et al., 1992; Wouterlood and Jorritsma-Byham, 1993; Dolleman-Van der Weel MJ et al., 1994; Brandt and Apkarian, 1992).

In this chapter the aim is to explore: (i) whether ENP grafts have more extensive projections into the host tissue than do primary tissue grafts post-transplantation and if so (ii) whether this may occur only in the xenograft paradigm.

6.2 Experimental Plan

The experimental design was structured so as to allow a comparison between allograft and xenograft environments as well as primary versus expanded tissues. This could only be performed systematically in our lab using rodent tissue which allowed both allo- and xenograft as well as primary and expanded tissue groups in a four way experimental design. However, we have a particular interest in human cells and therefore we proceeded with the xenograft arm alone in this particular experiment. Grafting human foetal tissue to rat in both the primary and expanded state would allow a direct comparison of cell type for the potential for long projections in the lesioned adult rodent brain and there is the advantage that there are effective and convenient human-specific markers for the identification of these cells in the host brain. Grafts of primary and expanded mouse donor cells into a mouse host comprised the allograft paradigm and were directly compared to the mouse grafts of primary and expanded mouse donor tissue into the rat host, i.e. a xenograft (Table 6.1).

Donor cells	Host animal	
	Mouse (adult lesioned)	Rat
GFP mouse Primary Tissue	√	√
GFP mouse 10day expanded ENPs	√	√
CD1mouse (LacZ) Primary tissue	√	√
CD1mouse (LacZ) 10day expanded ENPs	√	√
CD1mouse (Tracer) Primary tissue	√	√
CD1mouse (Tracer) 10day expanded tissue	√	√
Human Primary Tissue	X	√
Human 10day expanded ENPs	X	√

Table 6.1 Outlines the different transplant paradigms in which graft projections were analysed for the purpose of this experiment.

Tracers = BDA, Neurobiotin and PHA-L

6.2.1 Human and Mouse Tissue

Whole ganglionic eminences from three human foetuses were used in these studies, with post conceptional ages ranging from 55-75d (c. 8.0-10.5w) as determined by *in utero* ultrasound. Mouse tissue was obtained from E14 GFP transgenic mice and E14 CD1 mice.

6.2.2 Propagation of striatal ENPs

Coarse single cell suspensions of whole ganglionic eminence were prepared as described in Chapter 2. For *in vivo* study, human cells were treated in one of 2 ways: ***i***) Stored as primary cells overnight in *Hibernation medium* (Hibernate E, Gibco) at 4°C at a density of 500,000 cells/ml; or ***ii***) Expanded as ENPs for 10 days (Chapter 2). Mouse cells were treated in a similar fashion with the omission of the overnight hibernation as this was not required. Briefly, 200,000 cells/ml were seeded in B27 *proliferation medium* supplemented with EGF (20ng/ml), FGF-2 (20ng/ml) heparin (5µg/ml) ± LIF (10ng/ml) (Mouse cultures were not expanded in the presence of LIF). ENPs were fed, by replacing half the medium with fresh medium containing twice the concentration of B27, EGF, FGF-2 and LIF, every 3-4 days.

6.2.3 Characteristics of ENPs in vitro

The characteristics of striatal ENPs following 10 days propagation were assessed *in vitro* as described in Chapter 2. Briefly, spheres were dissociated to a coarse single cell suspension and plated onto poly-L-lysine-coated coverslips at a density of $10x10^4$ cells in 30µl differentiation medium. After 4-6 hours cells were flooded with 500µl

of differentiation medium and allowed to differentiate for 7 days prior to fixation. Cells were fed by replacing half the medium with fresh medium every 3 days.

6.2.4 LacZ labelling of cells in vitro

Primary and 10 day expanded cells were treated with LacZ prior to transplantation. Conditions for infection were optimised by varying the concentration of virus used (MOI=molecules of infection) and the time for which the virus was exposed to the cells. The optimum infection was with an MOI of 2 for 1hour and all cell suspensions were treated equally.

Indirect fluorescent immunocytochemisrty was performed using standard protocols (Chapter 2) with primary antibodies directed against β-III Tubulin (1:1000), β-Galactosidase (1:6000) and GFAP (1:1000). Fluorescent staining was visualised on a Leitz DRMB microscope, and cell counts performed at x40 magnification. Pseudocolour fluorescent images were obtained using Openlab 2.1 image analysis software.

6.2.5 Neural Transplantation

Mouse to mouse and mouse to rat:

For all xenograft experiments animals were administered with Cyclosporine A (CsA) on a daily basis starting one day prior to grafting. GFP transgenic mice were bred in house. E14 foetuses were dissected as described in Chapter 2 and tissue was treated in one of two ways: i) Primary tissue was immediately prepared for transplantation and ii) Tissue was expanded in culture for 10 days in the presence of FGF-2 and EGF. CD1 mice were used for all subsequent mouse experiments as time-mated C57BL/6

mice could not be obtained from suppliers due to difficulties breeding. For LacZ treated groups CD1 E14 foetuses were obtained as described for GFP tissue. In this instance both the primary and 10 day expanded tissue was treated with an MOI of 2, of a 2.4 x 10^9 titre equine lentivirus carrying the LacZ reporter gene, for 1 hour at 37°C. Animals in the tracer groups received transplants of primary and 10 day expanded E14 CD1 mouse tissue untreated. One week prior to perfusion, 11 weeks post transplantation animals received an iontophoretic injection of tracer to the graft site, as described in Chapter 2.

Human to rat

The primary graft group received intrastriatal grafts of primary human striatal tissue, hibernated over night. Following 10 days expansion in culture the second group of animals received an intrastriatal graft of cells. As with mouse grafts just described, the human grafted animals received iontophoretic injections of the anterograde tracers one week prior to perfusion. Human tissue was not treated with the Lac Z virus or with the anterograde tracers as it was considered that there were adequate antibodies available for the identification of human tissue grafts.

Control animals received grafts of dead cells and in the tracer groups control animals received an injection of tracer to the lesioned striatum to estimate the extent of non-specific labelling within the lesioned striatum when analysing the graft data.

All cell implants were performed as described in Chapter 2 and consisted of 2µl injections of 500,000 cell suspensions delivered over 2 mins. Briefly, for ENP grafts, an aliquot of the cells was dissociated, via a trypsin digest and mechanical trituration,

to single cells, allowing an estimate of the cell number and viability within the sphere suspension as well as allowing cells to be differentiated for *in vitro* characterisation. Using this information, undissociated spheres were harvested by centrifugation and re-suspended in DNase at 250,000 viable cells/μl. Hibernated/Primary tissue were prepared for transplantation by washing the tissue three times in DMEM/F12, determining the viable cell count and re-suspending the cells in DNase at a density of 250,000 viable cells/μl. All animals that received a xenograft were administered CsA daily starting one day prior to transplantation.

6.2.6 Iontophoresis

One week prior to perfusion animals received an iontophoretic injection of Neurobiotin, PHA-L or BDA to the graft site. The tracer was injected with a square wave pulse of 10 micro-amps at a rate of 7secs on 7secs off for 20 minutes with a micropipette of 20-50μm diameter.

6.2.7 Histology and immunohistochemistry

Twelve weeks following transplantation animals were transcardially perfused and their brains processed for histological analysis. Serial coronal 40μm frozen sections were prepared, collected and stored as described in Chapter 2. A 1:12 series of sections were processed for Nissl staining using cresyl violet. A further 1:6 series was processed for indirect single label immunohistochemistry with the following primary antibodies:

Mouse anti-HuNu (1:1500) (human specific nuclei)
NF70 (1:500)
Rabbit anti-β-GAL (1:6000)
Chicken anti-GFP (1:4000)
Neurobiotin
PHA-L
BDA

The basic protocol was identical in all cases and is described in Chapter 2. Visualisation was via the DAB method. Where double labelling was carried out the first antibody was completed before commencing the second antibody which was visualised using the Vector SG kit. Staining controls consisted of omission of the primary antibody and these confirmed the specificity of staining in all cases.

6.2.8 Quantification of graft parameters

Graft volume and cell number was determined as described (Chapter 2) on a 1:12 series of Nissl-stained sections. Fibre outgrowth from the graft was calculated by manual counts and in the case of human grafts where the fibre density was such that accurate counts could not be made an estimation of fibre number was made.

6.3 Results

6.3.1 In vitro characteristics of grafted cells

Striatal mENPs used for transplantation underwent a 5x increase whereas hENPs underwent a 1.6x increase in absolute cell number after 10 days, when characteristic spheres were seen in suspension. Primary tissue had viability above 95% in all cases based on trypan blue exclusion assay analysis. Cells labelled with the LacZ LV had a lower viability and to allow for this, double the volume of cells was exposed to the virus. Uptake of the virus was analysed by immunohistochemistry and found to be in the range of 60-70%.

6.3.2 Graft survival and morphology

A total of 192 animals received grafts with n=8 for each graft group. Graft survival in general was good. Mouse to mouse grafted animals had a 90% graft survival whereas mouse to rat graft survival was lower at 75%. Human to rat grafted animals had a 65% graft survival.

6.3.3. Graft volume and cell number.

Graft volume was measured based on Nissl stained sections. For analysis, mouse to mouse and mouse to rat graft groups for each different method of tracing were grouped together. Despite the transplantation of equal numbers of cells the volume of mouse donor grafts was generally small, whereas those of human grafts were large and tended to fill the entire striatum. There was no significant difference in the volume of primary and expanded mouse grafts however there was a significant difference between human graft volume and all other groups (Figure 6.1A,

$F_{2,192}$=562.31, p<0.001). The total cell number in each group was calculated based on Nissl stained sections, as described in chapter 2 and, as for graft volume, all mouse to mouse and all mouse to rat graft groups were combined. There was no significant difference in the number of cells in the mouse to mouse and mouse to rat graft groups, however there was a significant difference in the number of cells in the human grafts than all other graft groups (Figure 6.1B, $F_{2,192}$=372.95, p<0.001).

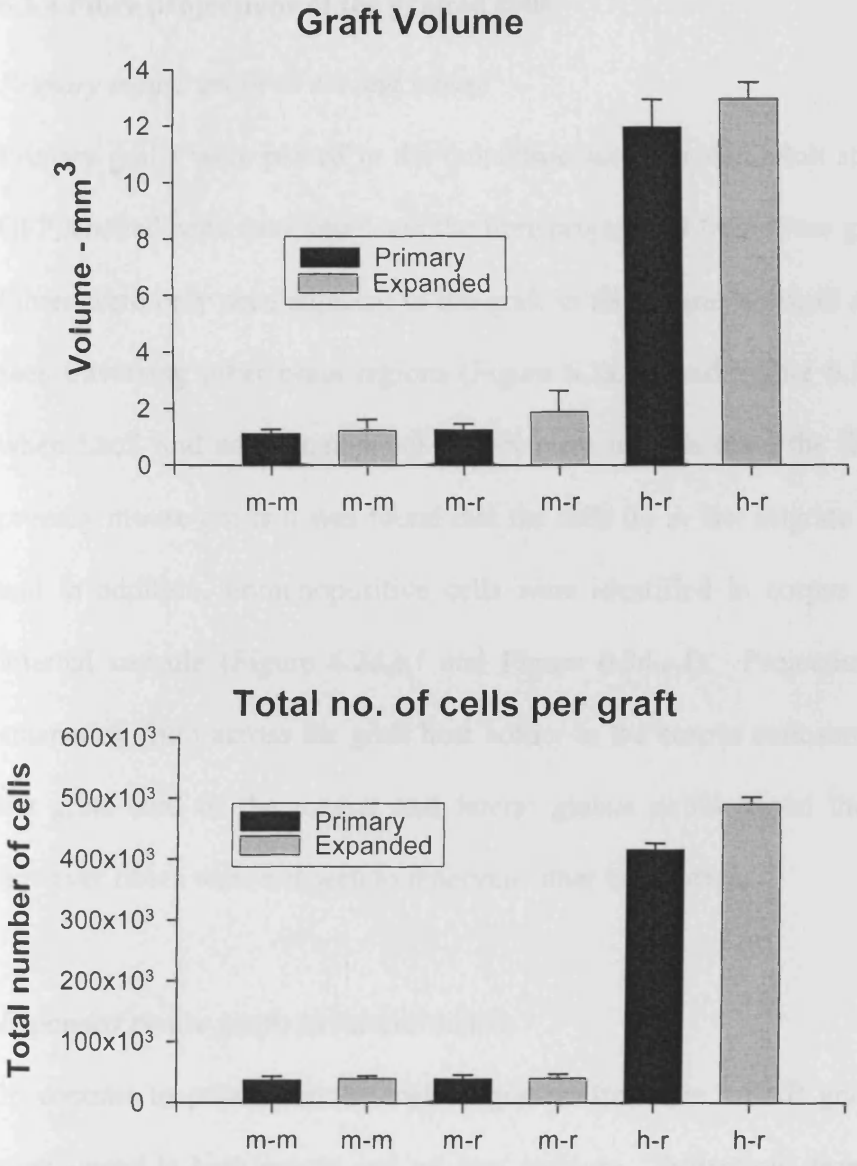


Figure 6.1 **A)** Graft volume revealed no significant difference between primary and expanded grafts. There was no difference between mouse to mouse and mouse to rat grafted animals. There was a significant difference in the graft volume of human to rat animals compared to all other graft groups. B) The total number of cells was calculated from Nissl stained sections and there was a significant difference between animals receiving grafts of human tissue compared to all other grafted animals. (m-m = donor mouse cells to mouse host, m-r = donor mouse cells to rat host, h-r = donor human cells to rat host)

6.3.4 Fibre projections of the grafted cells

Primary mouse grafts to rat and mouse

Primary grafts were placed in the quinolinic acid lesioned adult striatum. Grafts of GFP labelled cells were small and the fibre projections from these grafts were limited. Fibres were only seen adjacent to the graft in the striatal neuropil and no fibres were seen traversing other brain regions (Figure 6.2a,b,c and Figure 6.3a,b,c). However, when LacZ and neuroanatomical tracers were used to trace the fibre projections of primary mouse grafts it was found that the cells do in fact migrate out from the graft and in addition, immunopositive cells were identified in corpus callosum and the internal capsule (Figure 6.2d,e,f and Figure 6.3d,e,f). Projections were observed emanating from across the graft host border to the corpus callosum and further from the graft core to the medial and lateral globus pallidus and the internal capsule however fibres were not seen to innervate other brain areas.

Expanded mouse grafts to rat and mouse

In contrast to primary grafts, cell migration from the mENP graft core was more pronounced in both mouse and rat host striatum. Projections from expanded grafts were observed in similar areas to that of primary grafts, such as the corpus callosum, the internal capsule and the globus pallidus. However, fibres in these grafts were also observed in more distal regions such as the basal nucleus, the ventrolateral thalamic nucleus, the subthalamic nucleus, the entopeduncular nucleus and the basolateral amygdaloid nucleus. No fibres were seen in the substantia nigra of these grafts (Figure 6.4 and 6.5). The use of LacZ labelled cells and the neuroanatomical tracers allowed the identification of the graft fibres that were not observed in GFP grafted animals (Figure 6.6).

Primary grafts of human tissue to the rat

Primary human foetal tissue was transplanted to the lesioned rat brain. Grafted cells were identified with the human specific immune marker NF_{70} (Figure 6.7). Graft cells were located throughout the striatum and fibre projections were seen crossing the graft – host border to the corpus callosum as well as along the needle tract as a result of backflush during the grafting procedure. Fibres from human derived neurons were also observed in the globus pallidus and caudally in the subthalamic nucleus and entopeduncular nucleus.

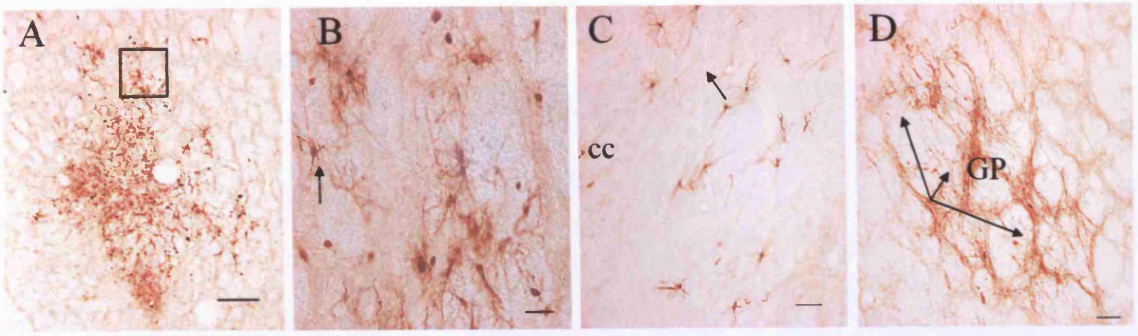
10 day expanded hENPs grafted to the rat

Human ENPs were expanded in culture for 10 days before grafting to the lesioned striatum. As above, these cells were identified with NF_{70}. Immune positive graft cells were located in the striatum with fibre projections emanating from the graft across the graft host border. Immune positive fibres were identified in the corpus callosum, and the globus pallidus, more caudally fibres were identified in the subthalamic nucleus, entopeduncular nucleus, and the substantia nigra.

Table 6.2 summarises the projections for each paradigm and also compare iontophoretically injected animals to GFP and LacZ labelled grafts.

Primary mouse tissue to rat host brain (xenograft)

GFP labelled primary mouse cells



LacZ labelled primary mouse cells

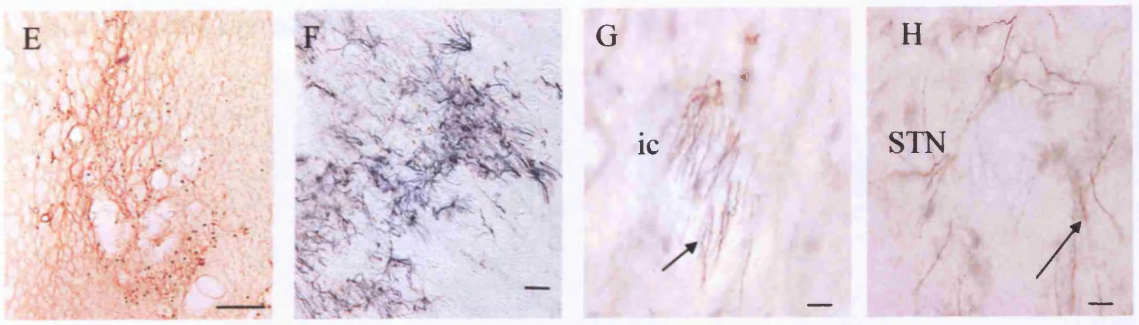


Figure 6.2 Primary xenografts of mouse striatal donor tissue to the lesioned rat adult brain. A and E, lower power magnification showing few fibre projections protruding from the graft core. B higher power of A shows GFP positive projections travelling relatively short distance (arrow) in contrast to F where lacZ labelled cells are shown to send out longer projections. Some GFP immune positive cells were seen in the corpus callosum (cc) and in the globus pallidus (GP) of one animal B and C (arrows). G and H show lacZ positive fibres in the internal capsule (ic) and the subthalamic nucleus (STN) (arrows). Scale bar (A and B) = 500μm, all others =200μm. I, represents a schematic of the summary of areas where projections (as shown by grey lines) were seen in all primary mouse grafts to the rat host brain.

Primary mouse donor tissue to the mouse host brain (allograft).

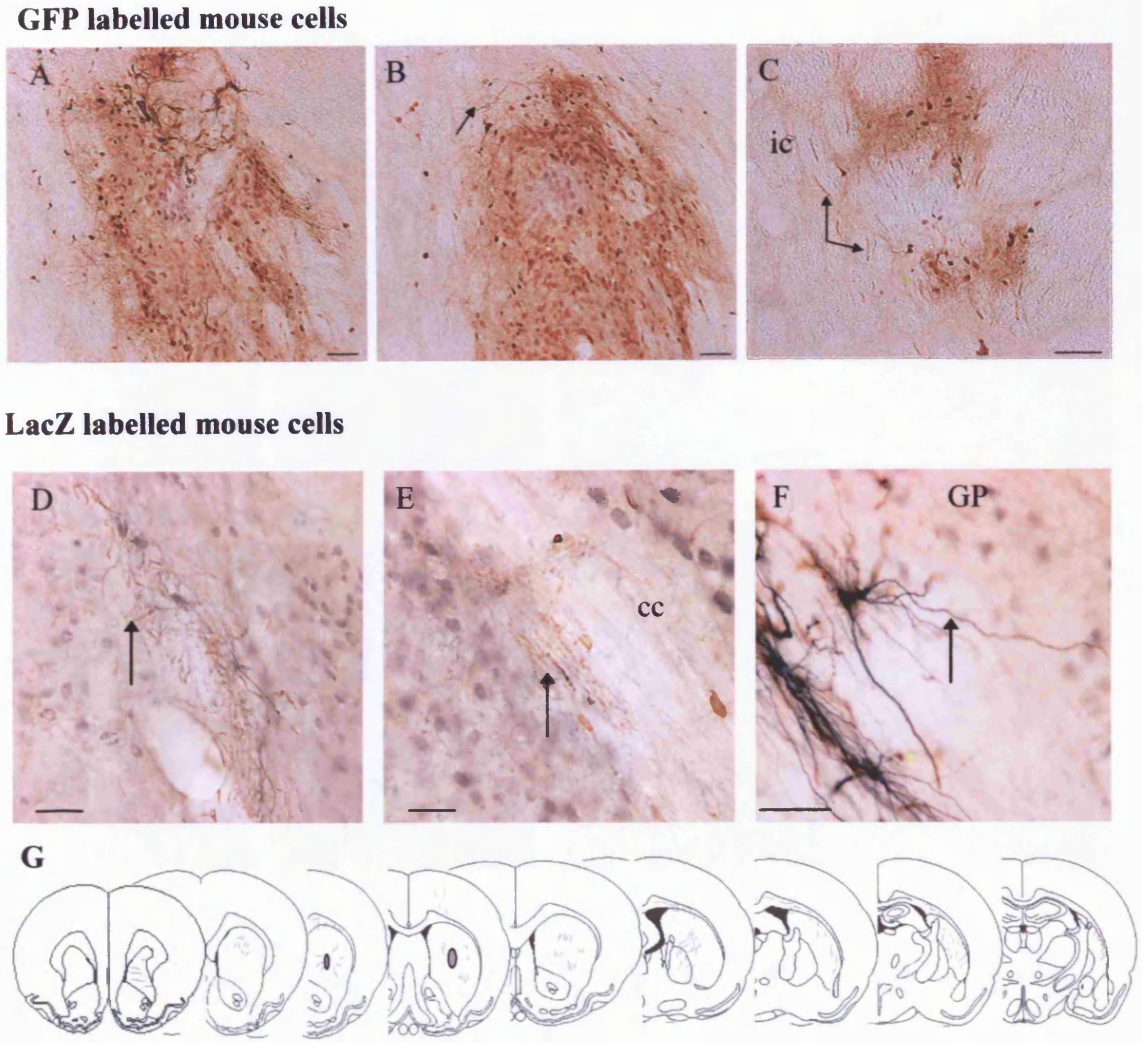


Figure 6.3 Primary graft of GFP (A-C) and LacZ (D-F) labelled striatal tissue to the lesioned adult mouse brain. This is the largest graft in the GFP graft group (A,B, show the graft mass) with all others being small thin grafts, similar to those shown in Figure 6.2 and 6.4. Positive cells and projections were seen in the striatum, D (arrow), the internal capsule (ic), (C) and the corpus callosum (cc) (E) as well as positive fibres in the globus pallidus (F) (arrows). Scale bar = 500μm (A-E) and = 200μm (F). G represents a schematic of the summary of areas where projections (as shown by grey lines) were seen in all primary mouse grafts to the mouse host brain.

Expanded mouse grafts in the rat brain (xenograft)

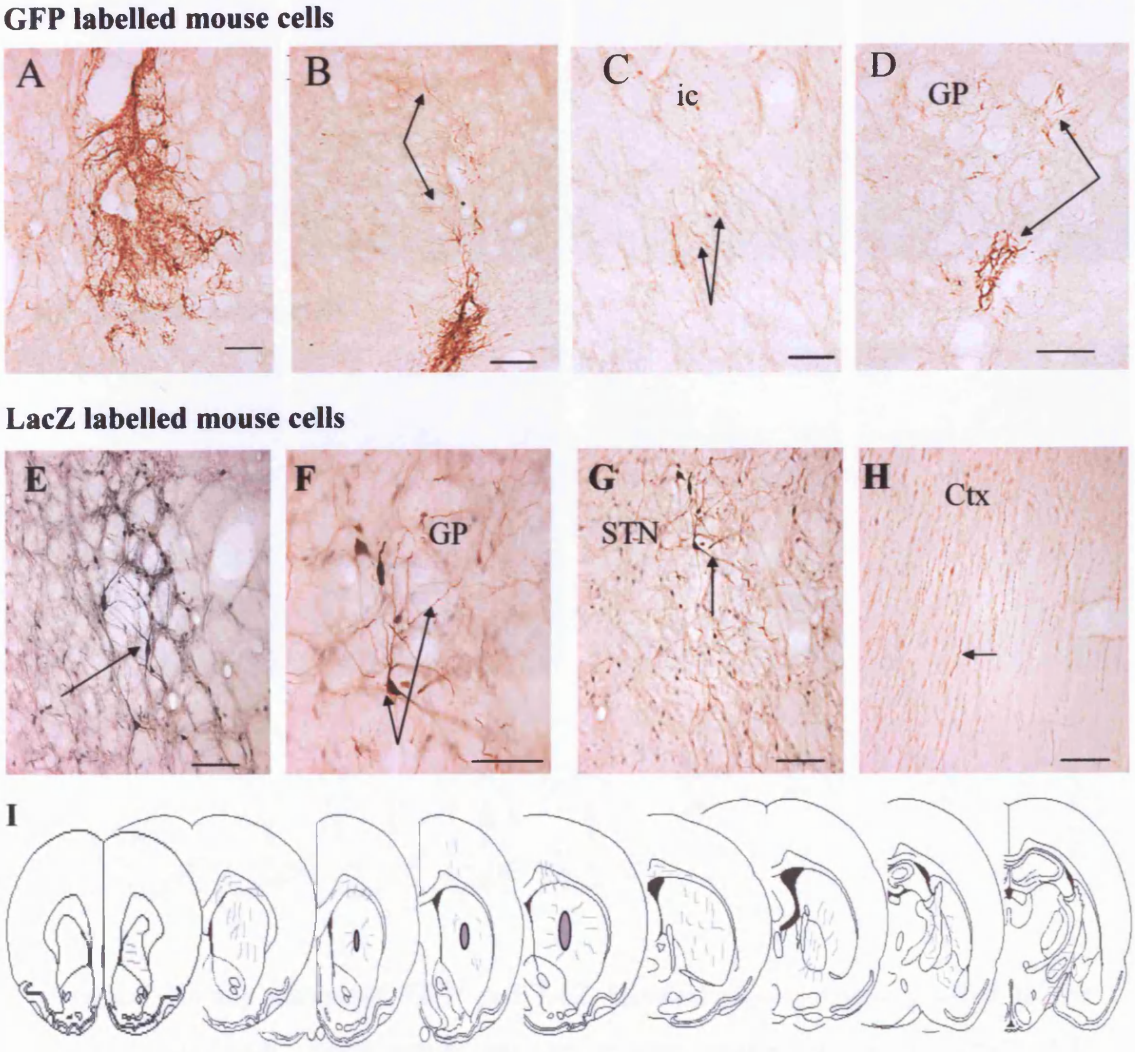


Figure 6.4 10 day expanded GFP and lacZ labelled ENPs grafted to the lesioned rat striatum. Projections can be seen emanating from the graft across the graft host border (A and arrow in B). GFP positive fibres were seen projecting in the internal capsule (ic), C and to the globus pallidus (GP), D. LacZ labelled cells and fibres were identified in the globus pallidus (GP), subthalamic nucleus (STN) and the cortex (Ctx) (arrows), H, and, G. Scale bar =500μm. I, represents a schematic of the summary of areas where projections (as shown by grey lines) were seen in all expanded mouse grafts to the rat host brain.

Expanded mouse grafts in the mouse brain

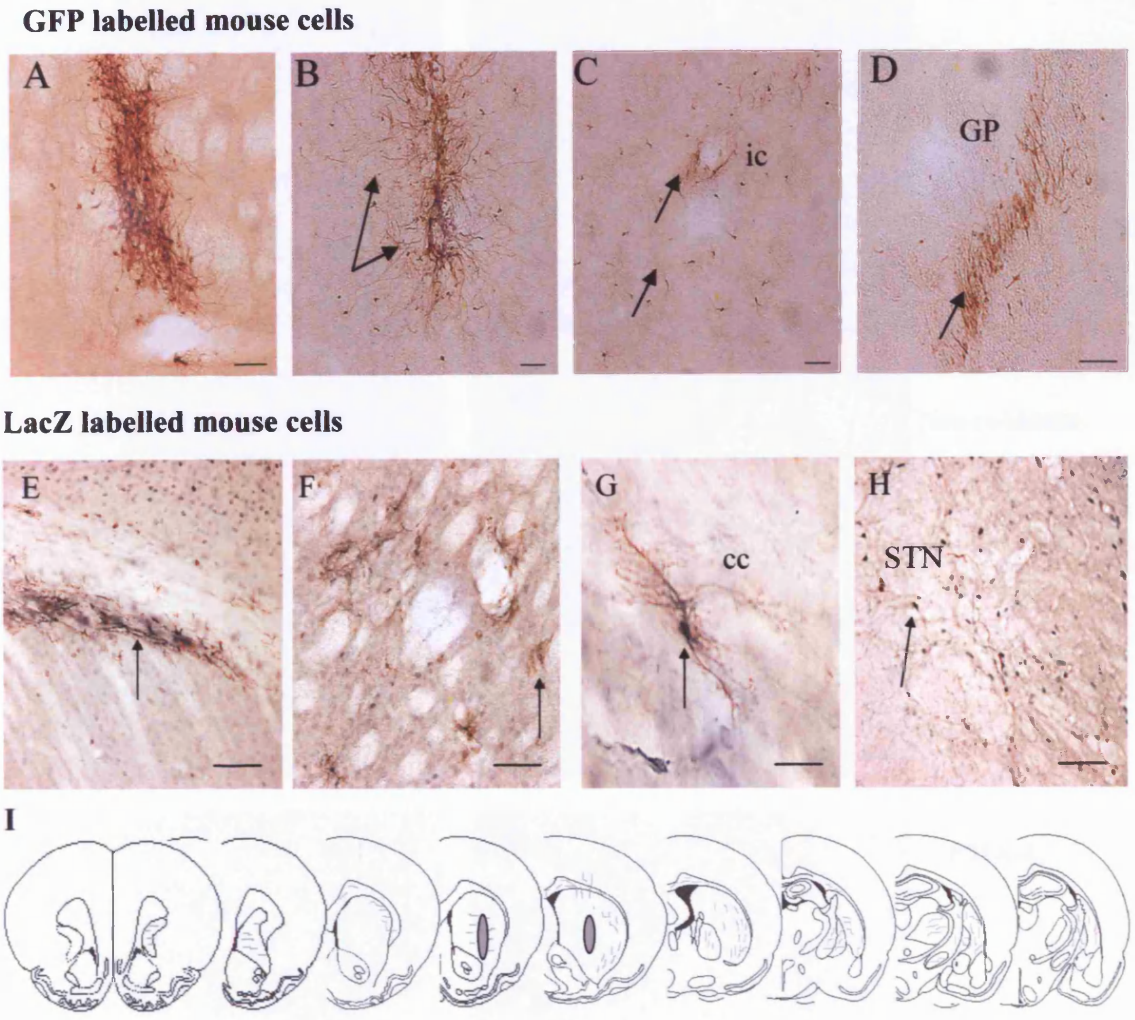


Figure 6.5 10 day expanded GFP and lacZ labelled striatal tissue grafted to the lesioned mouse brain. Fibre projections can be seen emanating from the graft (A,B and E) across the graft host border. GFP positive fibres were seen projecting in the internal capsule (ic) and to the globus pallidus (GP) (arrows). Immune positive fibres (arrow) were observed in the grey matter close to the graft, F. Dense axonogenesis of the grafted cells was observed on lacZ labelled cells, G (arrow). Scale bar = 500 (A and E) and 200 μm (all others). I, represents a schematic of the summary of areas where projections (as represented by grey lines) were seen in all 10 day expanded mouse grafts to the rat host brain.

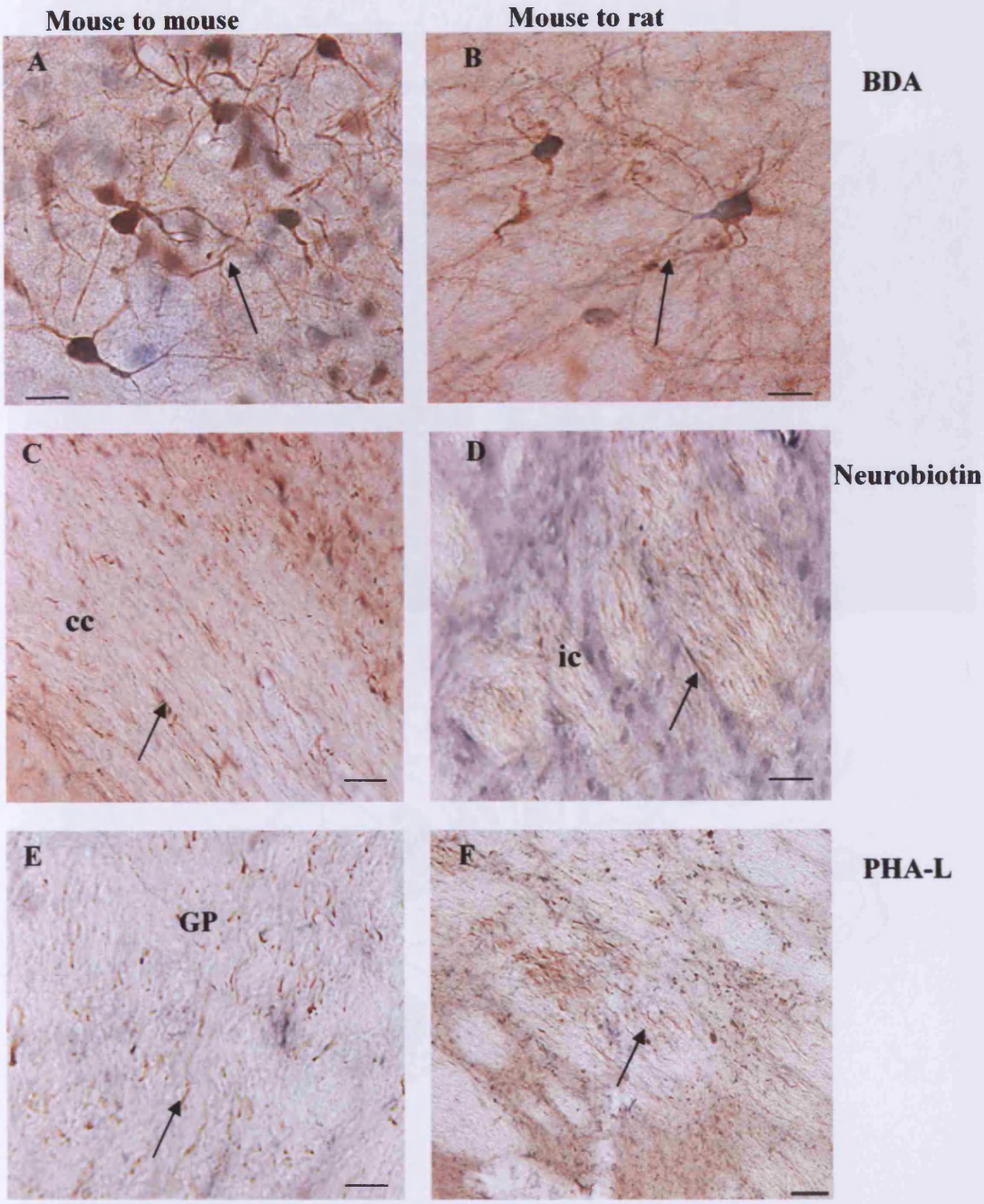


Figure 6.6 Anterograde tracers were inotophoretically injected to the graft region one week prior to perfusion. Immune positive fibres were identified for each tracer in a similar pattern to that of lacZ labelled cells and a sample of each tracer is shown here for clarity of presentation. A, and B represent BDA positive cells within the graft. C and D, neurobiotin positive projections in the corpus callosum (cc) and the internal capsule (ic). E and F, PHA-L positive projections in the globus pallidus (GP). There were no major differences in the pattern of projections between each labelled group. Arrows point to immune positive fibres. Arrows show immune positive projections. Scale bar = 500μm.

Human cells transplanted into the rat brain labelled with NF_{70}

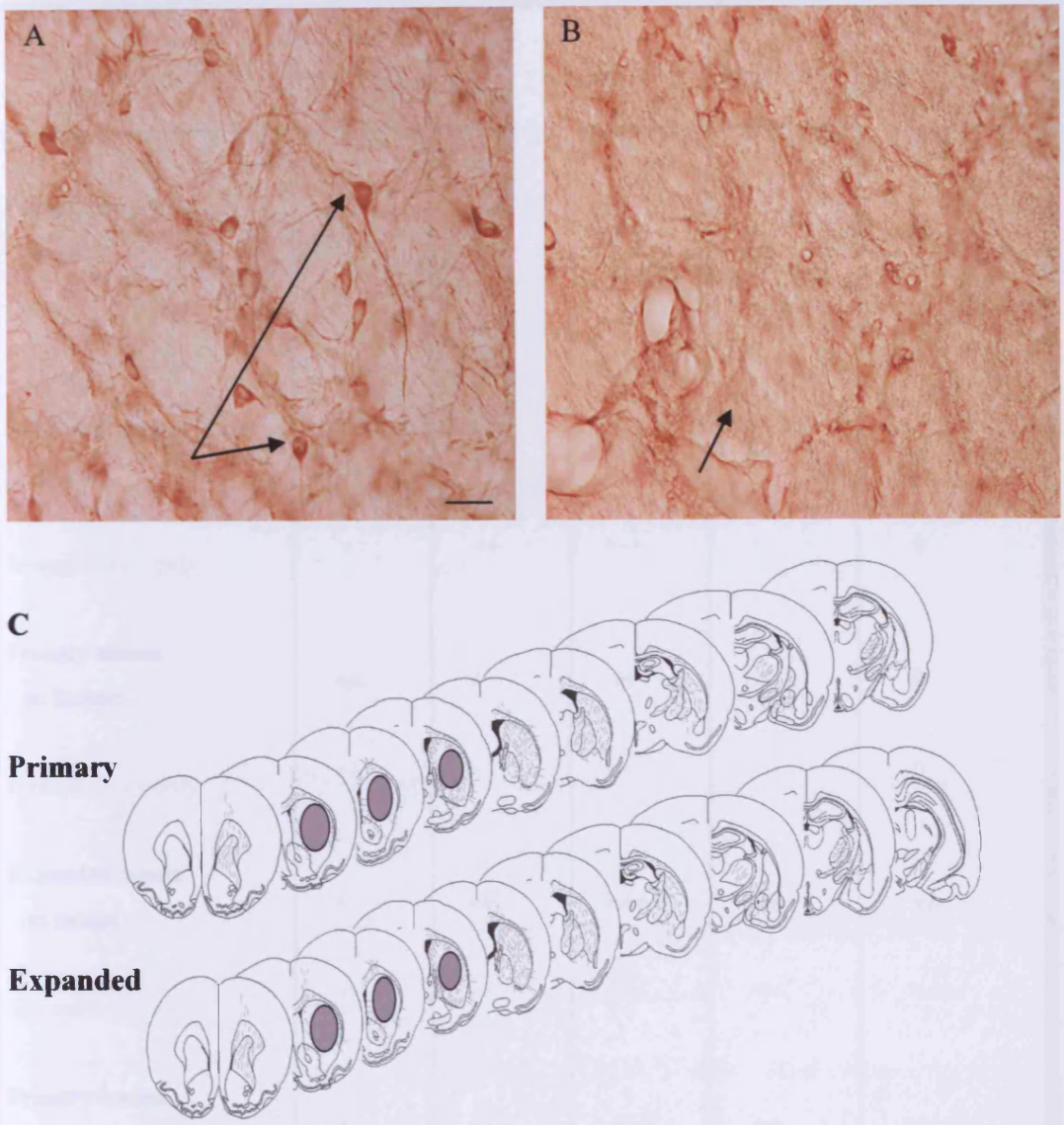


Figure 6.7 An antibody to NF_{70} was used to identify the human derived grafted cells in the host brains, A and B. Human primary and expanded grafts had a larger volume than mouse grafts with grafted cells acquiring the entire striatum, as represented in the schematic of human graft projections in the rat brain, C. Immune positive fibres were identified caudally in the subthalamic nucleus, the globus pallidus, and in substantia nigra as well as rostrally in the region of the olfactory bulb. C represents a schematic of the summary of areas where projections (as represented by grey lines) were seen in all primary and 10 day expanded human grafts to the rat host brain.

Table 6.2 Summary of fibre projections in the host brain using all approaches.

	CC	IC	GP	STN	SN
Primary mouse to rat	+/-	+	+++	+/-	0
Iontophoresis only	+/-	+	+++	+/-	0
Expanded mouse to rat	+	++	+++	+	+/-
Iontophoresis only	+	++	+++	+	0
Primary mouse to mouse	+/-	+	++	+/-	0
Iontophoresis only	+/-	+	++	+/-	0
Expanded mouse to mouse	+	++	+++	+/-	+/-
Iontophoresis only	+	++	+++	+/-	+/-
Primary human to rat	+	+++	+++	++	+
Expanded human to rat	+	+++	+++	+++	+

0 = no fibres, +/- = negligible fibres, + = tens of fibres, ++ = hundreds of fibres and +++ = thousands of fibres, observed in each region, based on estimated or actual counts. Iontophoresis only animals for the corresponding groups are shown in red.

6.4 Discussion

The results presented in this chapter and summarised in Table 6.2, confirm that primary foetal cells project into the host parenchyma including some distant sites in the host brain and that expanded ENPs show a more marked ability to do so. The projections from grafted cells were observed at distances both rostral and caudal to the graft core in all paradigms examined in this study.

Mouse primary and expanded cells grafted in the allograft and xenograft environment were seen to send projections to striatal targets possibly via the internal capsule fascicles such as at the level of the external pallidum, the globus pallidus, the subthalamic nucleus and the basal nucleus. Fibres were also observed in host white matter tracts in which branching of the fibres could be seen. No immunopositive fibres were seen to innervate the substantia nigra in the primary tissue grafted animals and a tiny number of fibres were observed in the substantia nigra of expanded grafts in the mouse and rat brain. These were only identifiable when cells were treated with LacZ prior to grafting or when a tracer was iontophoretically injected to the graft.

In contrast to this, human ENPs grafted to the rat brain were shown to send long projections from the graft to the olfactory bulb and specific target regions of the striatum including the substantia nigra. Both primary and expanded human grafts were much larger in volume than mouse grafts, even though the same number of cells was grafted in each case, thus suggesting that human foetal tissue continued to proliferate *in vivo* to a much greater extent than any proliferation that may have been ongoing in the rodent tissue grafts. The density of fibres from human tissue grafts

was such that quantifying the number of projections in specific regions was difficult and therefore an estimation of fibre number was made.

It has previously been shown that primary human tissue transplanted to the rat brain can send out extensive projections to the pallidum and the substantia nigra (Wictorin et al., 1990; Wictorin et al., 1992) and that the projections of xenografted tissue tends to be limited to the normal target sites of the donor tissue itself (Garcia et al., 1995; Wictorin et al., 1992; Isacson et al., 1995a; Isacson and Deacon, 1996). Armstrong (Armstrong et al., 2000) has previously shown that when primary human tissue was grafted to the lesioned striatum, projections were only observed as far as the pallidum whereas the fibre projections of expanded human ENP grafts were much longer and were present in non-specific targets of the host tissue. In this study such non-specific fibres were not observed to the same extent and immune positive fibre were only observed traversing the corpus callosum to the contralateral side however, expanded ENP grafts did present projections in more caudal regions of the host brain. It may be that there are specific guidance cues still present in the adult CNS that ENPs are responsive to in a manner not seen for primary tissue, thus allowing these cells to send out such long projections (Garcia et al., 1995).

Direct comparison of the graft groups would suggest that human derived grafts had a greater potential to send long projections into the host brain than those of mouse derived grafts. This raises the issue in relation to relative brain size: human tissue being derived from a phylogenetically more mature donor may have the necessary receptors for long distance growth which mouse derived cells would not have, given the differences in brain size of the two donors. Studies of pig tissue xenografted to

the rat brain also show the potential for long distance projections to traverse the host brain (Armstrong et al., 2003b; Armstrong et al., 2002) and therefore the use of xenografted human tissue into rodent host brains may not provide a true representation of the potential of this tissue for use in neural transplantation. Another possibility for the differences observed in this study may be due to the presence of specific signals in the allograft and xenograft brain. Xenografted donor cells may be unresponsive to specific stop signals thus leading to longer projections. However, the first would seem more likely in this case given that there were not the same fibre outgrowth observed from the mouse to rat grafts as that seen in human to rat grafted animals.

A further consideration is whether CsA could have an effect as all xenografted animals in this study were administered CsA throughout the experiment and allografted animals were not. CsA has been reported to have an effect on the neuronal growth *in vitro* (Steiner et al., 1997). However, a substantial effect is not likely as there were no substantial differences between the allo- and xenograft environments and thus it appears to be largely due to the expanded cells. Further studies in which all animals, both allo- and xenografted are immunosuppressed are required to be able to compare all graft groups directly as it is not clear what effects CsA may be having on the graft itself.

Another crucial issue with respect to the interpretation of these studies is cell migration. It was observed that there was migration of the primary as well as the expanded cells in the host brain although the degree of migration appeared to be greater in the animals receiving ENPs rather than primary tissue, however; this was

not quantified in this study. In a recent report (Hurelbrink and Barker, 2005) it was shown that allografted cells had the potential to migrate throughout the host brain and that human derived cells migrated significantly more than mouse or rat cells. Thus it may be that at least a proportion of the long projections observed in this study in the human to rat grafted animals could be a direct result of cells having migrated away from the graft core, thus having the potential to reach more caudal brain regions. In this respect, the study using iontophoretic injection of tracers to the graft site was informative as there were no projections observed in the more caudal nuclei such as the substantia nigra in most animals. This suggests that indeed, some of these projections may originate from cells that have migrated away from the graft core. Thus, there is the possibility that ENPs have the potential to project more extensively in the host brain, but it is clear that the migration of cells in the host brain is also a factor that needs to be taken into consideration. If migration was a crucial factor it would then suggest that the potential for projection outgrowth is not influenced entirely by specific striatal signals, as the cells that have migrated away from the striatum will be exposed to different signals than those within the striatum.

In this study, GFP labelled cells showed much less pronounced projections and this may be a result of down regulation of the transgene *in vivo*. It was found that, when compared to Nissl stained sections, only a proportion of the cells were labelled positive for GFP. Looking at GFP expression over time *in vivo* demonstrated that the GFP expression was down-regulated with time, and this is in accordance with Eriksson (Eriksson et al., 2003) who also reported a down-regulation of GFP expression albeit under a different promoter. Thus it may be that, at the time points analysed, this particular GFP transgene was significantly down-regulated in a large

proportion of cells and hence the difference in the fibre networks from these grafts compared to those labelled with the anterograde tracers and the lacZ labelled grafts. Details of the down regulation of GFP are shown in Appendix 4.

The use of the neuroanatomical markers in this study was to characterise further the fibre outgrowth of the allograft and xenografted mouse tissue grafts. Anterograde tracers were used in this study so as to trace the graft derived fibres in the host brain, given the non-specific nature of expanded graft fibres reported by Armstrong (Armstrong et al., 2000). Retrograde tracers were not employed in this study, although several studies of primary grafts have been carried out using such tracers and they have shown that fibres from the globus pallidus and entopenduncular nucleus project back to the graft and the labelled neurons were shown to overlap with patches of DARPP-32 and AChE positive staining (Wictorin et al., 1989b; Wictorin and Bjorklund, 1989; Wictorin et al., 1989c; Wictorin et al., 1989a). Neurobiotin, BDA and PHA-L are all neural anterograde markers that have been shown to clearly label neurons *in vivo* (Novikov, 2001). There was no significant difference in the labelling pattern of the three tracers although it was apparent that BDA and neurobiotin labelled the axons and dendrites in a more specific manner than did PHA-L. The immune positive fibres identified corresponded to those observed in lacZ labelled neurons. Other studies incorporating the use of anterograde tracers to label the donor primary derived projections in the host brain have also reported fibre outgrowth in the globus pallidus and in some cases the entopeduncular nucleus (Wictorin et al., 1989c; Pritzel et al., 1986). Where human tissue was the donor, immune positive fibres were also observed more caudally in the substantia nigra and the cerebral peduncle. To overcome the possibility of leakage of the tracers, and the subsequent possibility of

misconceptions in relation to the fibre projections of the graft tissue as opposed to host brain projections, control quinolinic acid lesioned animals received an injection of tracer, and labelling from these animals was then used to identify possible host derived projections in the grafted animals. In control animals there was no sign of labelling of projections as seen in grafted animals following iontophoretic injection of tracers.

Further studies looking at the behavioural differences between these primary and expanded graft groups are warranted to understand the functional significance of these fibre projections. However, such studies using human tissue are limited in the xenograft paradigm given the problems associated with graft survival and the ongoing need for daily immunosuppression. Therefore an alternative model system is required to overcome such issue (see Chapter 7).

We can conclude from this study that the outgrowth from expanded ENPs is greater than that of primary grafted tissue although as already mentioned further studies are required to address the issue of cell migration from these grafts as this may be the source of a proportion of the fibre outgrowth reported here.

Chapter 7

A new animal model to facilitate analysis of CNS xenografts.

Summary

Xenotransplantation of neural tissue to the host brain requires immunosuppression of the host to prevent graft rejection. However, this limits the amount of detail that can be obtained about the potential of the xenografted tissue, and of particular interest for the clinical work, human tissue. Thus, an animal model system is required that would allow long term analysis of these grafts *in vivo*. This chapter describes a novel animal model in which the animal is tolerised in the neonatal period, and consequently does not require immunosuppression, thus allowing long term graft analysis. Animals that received an ip injection of primary or expanded human tissue in the neonatal period subsequently supported intrastriatal grafts of the same tissue in the adult without immunosuppression. Animals that received an intrastriatal neonatal graft followed by an adult intrastriatal graft in the contralateral striatum resulted in poorer graft survival.

7.1 Introduction

Previous chapters have explored the potential of human neural precursor cells as an alternative for neural transplantation. One major limitation of these studies is the need for immunosuppression treatment of the host rodents to prevent rejection of the grafted human tissue. This involves daily injections of the immunosuppressant drug cyclosporine A (CsA), and without some form of immunosuppression grafts will be rejected within 4 weeks (Barker et al., 2000a). This drug is expensive and daily injections are unpleasant for the animals and inconvenient for the researcher. However, more importantly, it restricts the type of experiments that can be carried out due to the fact that the animals have limited tolerance of immunosuppression and become unwell after 12-16 weeks of treatment, thus restricting the ability to perform long term experiments for the assessment of the potential of human ENPs *in vivo*. This is crucial given that human ENPs take substantially longer to differentiate than rodent cells *in vivo*. An alternative to CsA is the use of immunocomprimised animals (e.g. Nude rats and SCID mice). These animals are immunocomprimised, with few if any T cells, to fight any form of infection and so must be housed in contained environments to reduce the risk of infections. Their lack of an immune system also renders them useless for detailed behavioural analysis following lesion and graft, as such studies impose a constant need for regular handling and removal from the contained air controlled housing and put the animals at severe risk of infection. As with CsA, nude rats are very expensive. Given these issues long term assessment of xenografted tissue has been limited, with approximately 20 weeks being the upper limit (Ostenfeld et al., 2000; Pundt et al., 1996c; Svendsen et al., 1997a). Thus it is clear that an alternative model is required in order to facilitate full assessment of the

true potential of human tissue for neural transplantation using long term evaluation of the grafts.

It has been shown that xenogenic tissue grafted into the CNS of non-immunosuppressed rodents in the neonatal period is not rejected, as the animals immune system is still developing until the latter part of the first week (Pundt et al., 1996b). Transplantation of human ENPs into the CNS of neonatal rodents has previously been carried out (Betarbet et al., 1996; Demeter et al., 2004; Lundberg et al., 2002; Munoz-Elias et al., 2004; Winkler et al., 1998; Young et al., 2000; Zigova et al., 2000; Englund et al., 2002a; Rosser et al., 2000; Olsson et al., 1997; Zigova et al., 2002). Olson (Olsson et al., 1997) transplanted E13.5-14 mouse LGE, MGE, and cerebellar primordium to the striatum of P1, P7 and P21 rat pups. P1 and P7 grafts were seen to integrate into the host brain and send out projections to striatal targets. P21 transplanted animals received daily immunosuppression with cyclosporine A to prevent transplant rejection. The transplants in P21 animals were similar to that reported for adult grafts in that the cells showed little migration but rather clustered around the injection site and the fibre projections from these cells were only reported in the globus pallidus. It was clear from this study that transplanting in the neonatal period can be beneficial for circuit reconstruction. The ability for these transplants to send out projections to striatal targets may be a result of specific guidance cues being expressed at this developmental stage or, may relate to the smaller size of the host brain at this time point, thus the donor cells have a shorter distance to travel in the host brain to reach specific target areas. However, the fact that the neonatal rodent brain is still undergoing growth and development means that signals are present that are not present in the adult brain. Thus, although it has proven an important model for

understanding the capacity of stem cells to respond to developmental cues, it is not an appropriate model to understand adult CNS transplantation.

The fact that human xenografted tissue survives in the neonatal brain into early adulthood suggests that the host rat may develop tolerance to the tissue. This study has explored whether tolerance can be induced in neonatal models using human foetal tissue that will allow a graft to be placed intracerebrally in adulthood without any form of immunosuppression. This requires (i) demonstrations as to whether tolerance developed to the transplanted tissue in the neonatal stage is lasting (i.e. do transplants alone in the neonatal brain survive long term into adulthood) and (ii) exploration as to whether injection of human tissue in the neonate will produce tolerance that will allow survival of xenogenic CNS tissue transplanted in adulthood.

7.2 Experimental Procedures

7.2.1 In vitro experiments

7.2.1.1 *Human tissue*

Cortical tissue from six human foetuses were used in these studies, with post conceptional ages ranging from 55-77d (c. 8.0-11.0w) as determined by *in utero* ultrasound.

7.2.1.2 *Propagation of cortical ENPs*

Coarse single cell suspensions of cortical tissue were prepared as described in Chapter 2. For the *in vivo* study these cells were treated in one of two ways: **1)** Stored as primary cells for up to 24hours in hibernation medium (Hibernate E, Gibco) at 4°C at a density of 500,000 cells/ml; **2)** Expanded in culture as ENPs for 10 days (Chapter 2). Briefly, 200,000 cells/ml were seeded in B27 proliferation medium supplemented with FGF-2 (20ng/ml), EGF (20ng/ml), LIF (10ng/ml) and heparin (5μg/ml). ENPs were fed by replacing half the medium with fresh medium containing twice the concentration of B27, FGF-2, EGF and LIF every 4 days. After 10 days propagation in culture these cells were either: **1)** transplanted to animals or **2)** frozen down as described in Chapter 2 for further use 6 weeks later.

7.2.2 In vivo experiments

7.2.2.1 *Characteristics of ENPs in vitro*

The characteristics of cortical ENPs following 10 days propagation were assessed *in vitro* as described in Chapter 2. Briefly, spheres were dissociated to a coarse single cell suspension and plated onto poly-L-lysine-coated coverslips at a density of $10x10^4$

cells in 30µl differentiation medium. After 4-6 hours cells were flooded with 500µl of differentiation medium and allowed to differentiate for 7 days prior to fixation. Cells were fed by replacing half the medium fresh medium every 3 days.

Indirect fluorescent immunocytochemisrty was performed using standard protocols (Chapter 2) with primary antibodies directed against β-III Tubulin (1:1000) and GFAP (1:1000). Fluorescent staining was visualised on a Leitz DRMB microscope, and cell counts performed at x40 magnification. Pseudocolour fluorescent images were obtained using Openlab 2.1 image analysis software.

7.2.3 Experimental Design

Primary tx into adult host CNS	10 day expanded tx into neonatal host CNS	10 day expanded tissue to neonate CNS	10 day expanded tissue injected ip to neonate	Primary foetal tissue injected ip to neonate	
↓	↓	↓	↓	↓	6weeks
		contralateral intrastriatal tx of same cells (cryporeserved)	adult intrastriatal tx of same cells (cryporeserved)	adult intrastriatal tx of same cells, primary tissue (different donor)	
		↓	↓	↓	6-12 weeks
Animals sacrificed and their brains processed for histological examinataion					
Tx = transplantation ip = intraperitoneal					

Table 7.1 Experimental plan.

Neonatal hosts were obtained from five pregnant Sprague-Dawley rats. No groups were given immunosuppression. Each neonatal animal was placed into one of five groups:

1. To receive a graft of primary human CNS tissue to the adult unilateral striatum for 20weeks without any form of immunosuppression.
2. To receive a graft of 10 day expanded human tissue to the neonatal striatum or hippocampus at P0 with subsequent survival for 12 weeks.
3. To receive a graft of primary or expanded human tissue to the neonatal striatum followed by a graft to the contralateral striatum at 6weeks with subsequent survival for 6 weeks.
4. I.P. injection of primary foetal cortical cells at P0 followed by a graft of primary foetal cortical cells from a different donor, to the striatum at 6-9weeks with subsequent survival for 6 or 13 weeks.
5. I.P. injection of 10d expanded human foetal cortical cells at P0 followed by a graft of the same 10d expanded human foetal cortical cells to the striatum at 6-9weeks with subsequent survival for 6 or 13 weeks.

7.2.2.2 *Neural Transplantation*

Group one (n=6) received intrastriatal grafts of primary human cortical tissue without any immunosuppression. On the day of birth all other animals received either an intrastriatal transplant of 10d expanded ENPs (n=18) or an intraperitoneal injection of primary cortical tissue (n=12) or an intraperitoneal injection of 10d expanded ENPs (n=12). 6-9 weeks after the initial transplant animals that had received a graft of 10 day expanded tissue to the striatum received a contralateral intrastriatal graft of the same 10 day expanded cells that had been frozen down after the initial transplant (n=9). The remaining animals in this group (n=9) did not receive a second graft. Animals that received an ip injection of primary foetal tissue received an intrastriatal transplant of primary foetal tissue (obtained from a different donor to that used in the initial injection) (n=12). Animals that received an ip injection of 10 day expanded cortical tissue as neonates received an intrastriatal transplant of the same cells (cryopreserved) 6-9 weeks later (n=12).

All cell implants were performed as described in Chapter 2 and for the intrastriatal transplants consisted of 2μl injections of 500,000 cell suspensions delivered over 2 mins. The ip injections consisted of a 1μl suspension of 200,000 cells delivered over 2 mins.

ENPs were prepared for transplantation after 10 days in culture. An aliquot of the cells was dissociated, via a Trypsin digest and mechanical trituration, to single cells, allowing an estimate of the cell number and viability within the sphere suspension as well as allowing cells to be differentiated for *in vitro* characterisation. Using this information, undissociated spheres were harvested by centrifugation and resuspended

in DNase at 250,000 viable cells/μl. Hibernated cells were prepared for transplantation by washing the cells three times in DMEM/F12, determining the viable cell count and resuspending the cells in DNase at a density of 250,000 viable cells/μl.

7.2.2.3 Histology and immunohistochemistry

Six to thirteen weeks following the second transplant animals were transcardially perfused and their brains processed for histological analysis. Serial coronal 40μm frozen sections were prepared, collected and stored as described in Chapter 2. A 1:12 series of sections were processed for Nissl staining using cresyl-fast violet. A further 1:6 series was processed for indirect single label immunohistochemistry with the following primary antibodies:

Mouse anti-HuNu (1:1500) (human specific nuclei)

Mouse anti-rat CD8 (1:500) (host cytotoxic T-cells)

OX42 (activated microglia)

Mouse anti-rat C3 (1:500) (host complement)

The basic protocol was identical in all cases and is described in Chapter 2. Visualisation was via the DAB method. Staining controls consisted of omission of the primary antibody and these confirmed the specificity of staining in all cases.

7.2.2.4 *Quantification of graft parameters*

Graft volume was determined as described (Chapter 2) on a 1:12 series of Nissl-stained sections. The extent of immunostaining for C3, CD8 and OX42 was graded on a scale according to the following strictly defined criteria, adapted from Larsson

and colleagues (Larsson et al., 1999a). For this procedure, coded sections were viewed and rated blinded to experimental groups and this is shown in Table 7.2.

Grade	*Histological criteria*
0[a]	No immunopositive cell present in the grafted striatum
1	Very occasional scattered immunopositive cells
2	Several positive cells, principally confined to peripheral graft regions or discrete patches, little perivascular distribution.
3	Numerous immunopositive cells present throughout the graft mass, perivascular distribution often obvious.
4	Florid infiltrate of immunopositive cells throughout the entirety of the graft mass, and often in the surrounding striatum.

Table 7.2 Histological criteria used to assess the degree if which markers of the host cellular and humoral immune response, CD8, OX42 and C3 were recruited. [a]A category of Grade 0.5 was introduced which was reserved for specimens where only a scar remained and the only immunpositive cells were occasional, rounded macrophage-like cells at the site of the scar.

7.3 Results

7.3.1 Cell status at transplantation

Cortical ENPs used for transplantation underwent an average 1.94 fold increase in absolute cell number after 10 days, at which time characteristic spheres were seen in suspension. Tissue maintained in hibernation medium for up to 24 hours maintained viability above 92% in all cases based on trypan blue exclusion assay analysis.

Subsequent *in vitro* experiments were undertaken in an attempt to further elucidate the identity of cells that composed the ENP population after 10 days expansion. A similar expansion in overall cell number was achieved in each donor expansion (2.5 ± 0.3 fold in total cell number). Cells could be induced to differentiate by removing the growth factors and plating on a substrate. By seven days, the cortical ENPs differentiated to yield a mixed population of β-III tubulin positive neurons (42.8% ± 7.0) and GFAP-positive (13.6% ± 3.6) astroglia, the remainder of the cells were not stained by either marker and their identity remains undetermined (Figure 7.1).

In vitro staining of differentiated primary and 10 day expanded human cortical tissue.

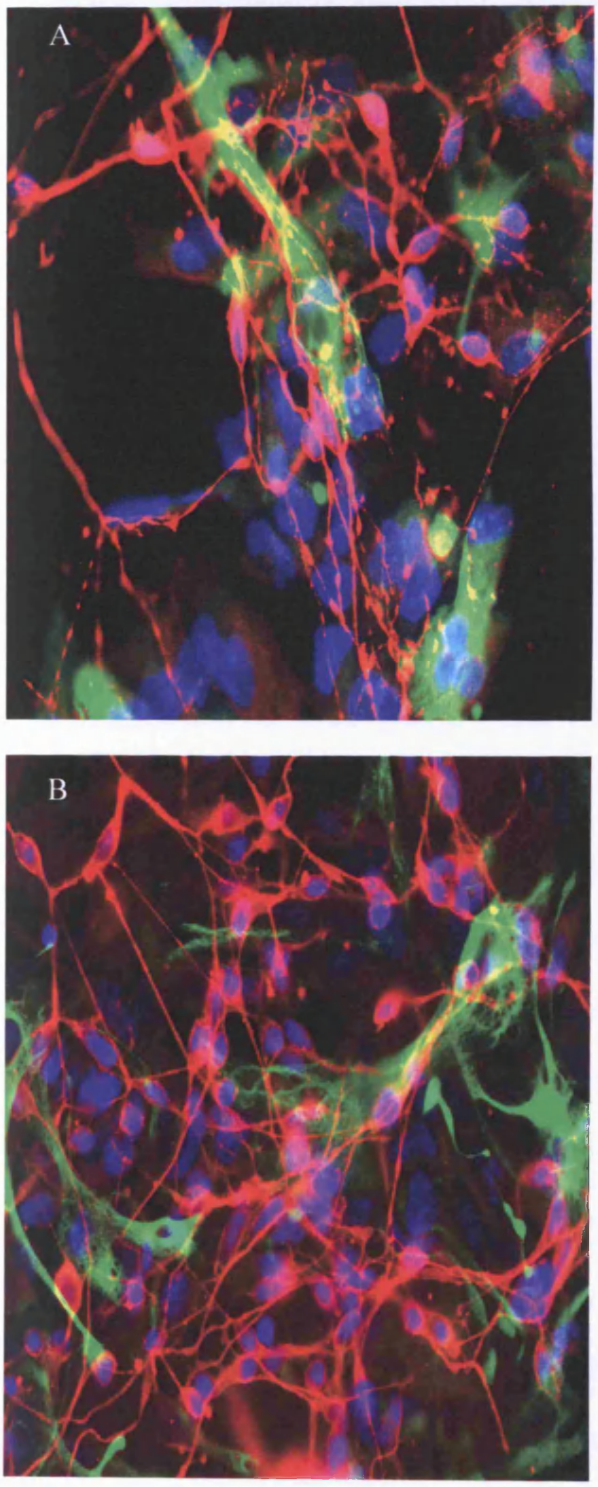


Figure 7.1
The *in vitro* characteristics of human cortical primary tissue (A), and 10 day expanded human cortical ENPs (B). β-III tubulin positive cells were present in both primary (64%) and expanded (42.8%) cell cultures (Red). There was an increase with time in culture in the proportion of GFAP positive astrocytes (Green) from 5.6% to 13.6%. Cell nuclei were labelled with Hoechst stain (blue). Scale bar 200μm

7.3.2 Graft survival and morphology

Surviving grafts could be identified in Nissl-stained sections (Figure 7.2 and 7.3) on the basis of cytological and cytoarchitectonic features and in sections stained for human specific antigen HuNu (Figure 7.4). Grafts of primary tissue transplanted into the adult rat CNS with no immunosuppression were rejected by 6 weeks post transplantation and there was no trace of any surviving cells. Nine animals received a unilateral intrastriatal graft of human cortical tissue at P0 and no further grafts were received. After 12 weeks survival 66% (6/9) of the animals appeared to have healthy surviving grafts. Of the 9 animals receiving an intrastriatal graft at P0 followed by a second intrastriatal graft as an adult only 44% (4/9) had surviving grafts 6 weeks after the second graft was received. These grafts were significantly smaller in volume when compared to all other groups (Table 7.3) ($F_{4,21}$=21.34,$p<0.001$). Animals that received an ip injection of either primary or 10 day expanded cells at P0 with a subsequent intrastriatal graft in adulthood had surviving grafts up to 13 weeks after the graft was implanted (93 and 50% respectively). The grafts are laterally placed but contain HuNu positive cells and show little sign of rejection (Figure 7.4) as measured by expression of the immunological markers OX42, CD8 and C3. Overall, there is a significant difference in graft survival between the grafts of 10 day expanded human foetal tissue and primary human foetal tissue ($F_{1,21}$=3.13, $p<0.001$) with the primary grafts showing optimum survival and this may be due to the large cell loss observed as a result of the freeze/thawing of the expanded cells which ultimately resulted in a smaller volume of cells being transplanted to the adult animal and hence the smaller volume of the graft. Where primary human foetal tissue was used as the tissue source there was no freeze/thawing of the tissue and as a result tissue used for the ip injection in the neonate was from a different foetus and in some cases of different gestational

age to that used for the intrastriatal adult graft. This appeared to have no effect on survival of the intrastriatal graft in adulthood.

Graft morphology was similar for primary and expanded grafts with both graft groups comprising a heterogenous population of cells and there was no apparent vascular infiltration or ventricular compression within the grafts. Primary grafts comprised of a heterogenous distribution of large neuronal type nuclei and smaller spindle-shaped nuclei (Figure 7.2 a,e and 7.2 c,g). 10 day ENP tissue grafts were densely cellular and positioned along the lateral wall of the caudate-putamen. The cellular distribution within the grafts was heterogenous, with zones of high cellularity interspersed among less intensely cellular regions (Figure 7.2 b,f). In animals with no discernible surviving grafts, only a thin strip of scar-like tissue was apparent at the level of the needle tract (Figure 7.2 d,h).

7.3.3 Graft Volume

Intrastriatal implants at P0 that were not followed by any other intervention resulted in significantly larger graft volumes than for any other group (t_4=7.02, $p<0.001$) (Table 7.3). In the group receiving a neonatal, followed by an adult intrastriatal graft, only four animals showed signs of surviving grafts on both sides. In all four cases both grafts were extremely small and there were increased signs of rejection with cells within the graft area immunopositive for the immune markers OX42, CD8 and C3 (Figure 7.2 c,g).

There was no significant difference in the volume of primary and 10 day expanded ENP grafts placed into animals that received an ip injection of human tissue at P0,

however both the primary and expanded grafts in this group had significantly greater volume than that of the group receiving a neonatal intrastriatal graft followed by an intrastriatal graft in the contralateral striatum in the adult (t_4=3.45, t_6=4.27, $p<0.001$) (Table 7.3).

Graft survival and volume measurements for each group.

Group	% Survival	Graft volume (Including rejected grafts)	Graft volume (Excluding rejected grafts)
Intrastriatal as neonate only	65%	4.4 ±0.1 mm^3	5.6 ±0.12 mm^3
Intrastriatal as neonate + contralateral striatum as adult	44%	**Neonate** 0.43 ±0.17 mm^3 **Adult** 0.35±0.15 mm^3	0.612±0.13 mm^3 0.504±0.11 mm^3
i.p as neonate + intrastriatal as adult	**ENPs** 50% **Primary** 93%	**ENPs** 1.9±0.08 mm^3 **Primary** 2.54±0.15 mm^3	2.85±0.1 mm^3 4.32±0.13mm^3

Table 7.3

Graft volumes and striatal volumes were calculated as described in Chapter 2 and are presented as mean ± SEM in this table. In the second column measurements have been determined for all animals within a group, whether or not a graft was considered to have survived and in the third column only for surviving grafts. There is a significant difference in graft volume between the group receiving 2 grafts (one in the neonate period and one in the adult period) and all other graft groups $P<0.001$. There is no significant difference in the striatal volume between groups.

Cresyl violet staining showing graft survival and morphology.

Typical morphology of intrastriatal grafts from primary and 10 day expanded ENPs in tolerised animals.

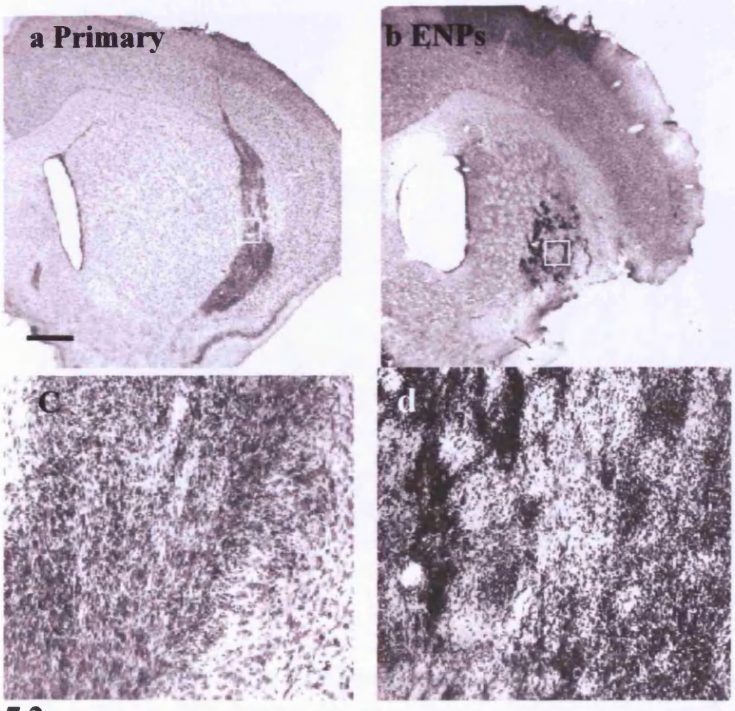


Figure 7.2
Graft morphology as demonstrated with Nissl staining. (**a,** and boxed detail **c**) the typical appearance of a primary grafts which have a characteristic graft core. In this example there has been some reflux of cells along the needle tract and the graft is laterally placed in the striatum. ENP grafts (**b,** and boxed detail **d**) in which the graft has a typical patchy appearance of ENP grafts. Scale bar = 500μm.

Neonatal intrastriatal and contralateral adult intrastriatal grafts of 10 day expanded tissue at P0 and adulthood.

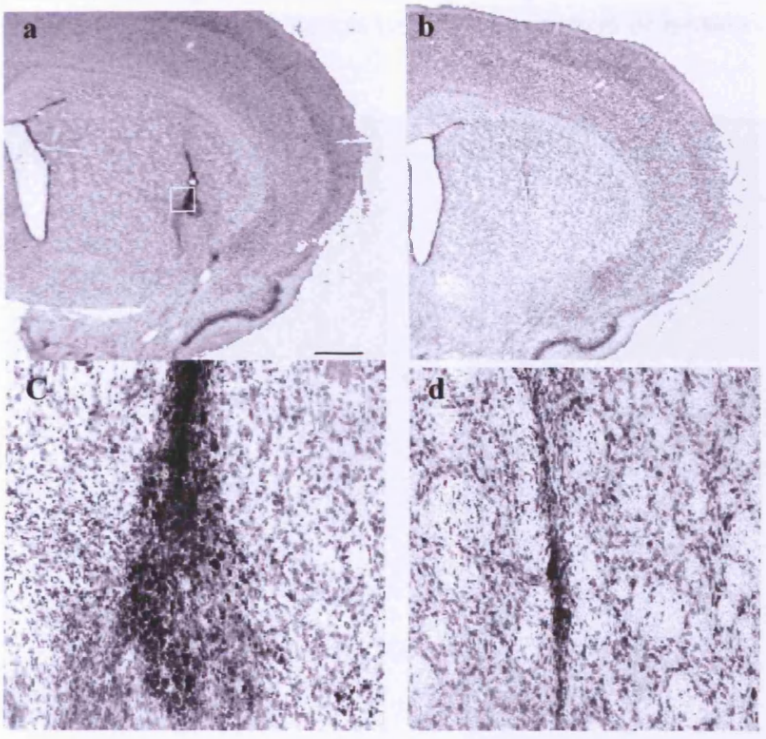


Figure 7.3
Grafts in the group receiving a neonatal intrastriatal and contralateral adult intrastriatal graft (**a,** and boxed detail **c**) were significantly smaller on both the right and left striatum than all other grafts. A glial scar signified a rejected graft (**b,** and boxed detail **d**). Scale bar 500μm (boxes are to scale).

HuNu positive staining in primary and 10day expanded human cortex grafts after 12 weeks *in vivo* following tolerisation with an ip injection of human cortical cells.

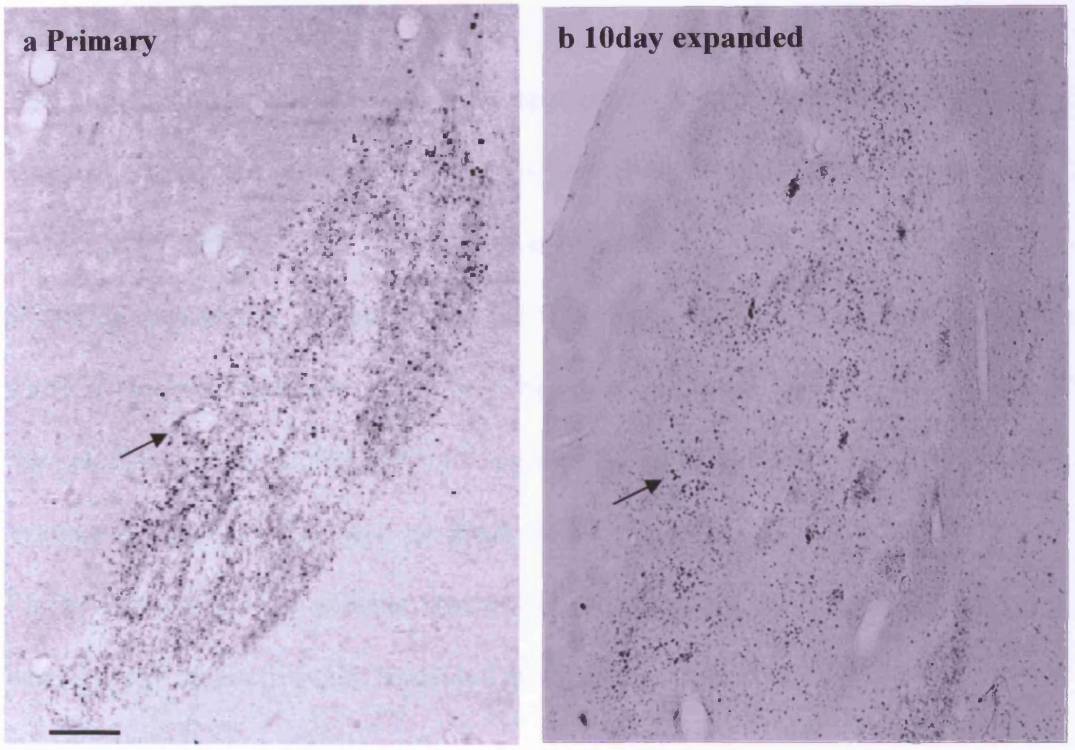


Figure 7.4
HuNu positive cells (arrow) represent human positive cells within the graft, a primary tissue graft **a)**, and a 10 day expanded graft **b)**, 10 day expanded graft showed more signs of migration out of the graft core to the surrounding neuropil whereas in the primary graft the HuNu positive cells are located mainly within the graft core. Scale bar 500μm

7.3.4 Host cellular immune response

In 'healthy surviving' grafts only very occasional, scattered CD8-positive cells were seen. The cells had a morphology consistent with them being lymphocytes: they were rounded with a high nucleus:cytoplasm ratio and CD8 staining had a cytoplasmic, punctuate distribution (grade 1, Figure 7.5a,e). The presence of signs of increasingly severe rejection correlated with increasing numbers of CD8-positive infiltrating cells. Such cells were predominantly confined to peripheral regions and small patched areas within grafts that showed a moderate sign of rejection. There was also evidence of clustering around the vasculature (grade 2, Figure 7.5b,f). Grafts which exhibited signs of marked rejection were very heavily infiltrated, and in such grafts CD immunoreactivity was often seen throughout the graft mass in cells with ramifying cytoplasmic processes as well as those with a lymphocytic appearance (grade 3-4, Figure 7.5c,d,g,h). In animals where the graft had not survived, only occasional weakly immunoreactive cells remained around the scar (Figure 7.5 d,h).

Activated microglia in the host were quantified using the same criteria as with CD8. OX42 positive cells in 'healthy' grafts were predominantly along the needle tract, and very few immunopositive cells were present within the graft core (grade1 Figure 7.6a,e). With increased rejection there was also an increase in the degree of OX42 immunopositive cells present in the graft core as well as a more pronounced infiltrate along the needle tract (grade 2, Figure 7.6b,f). Clusters of cells were seen in the graft core. Rejected grafts were heavily infiltrated with OX42 positive cells and this immunopositivity was present throughout the graft mass (grade 3-4, Figure 7.6c,d,g,h).

7.3.5 Host humoral immune response

Levels of C3 binding were generally very low and C3 was seen to bind most strongly at the edges of the grafts and around zones of necrosis and cavitation. Most animals showed little or no signs of C3 binding with only 3 animals showing high immunoreactivity with staining seen throughout the grafted area (Table 7.6, Figure 7.4).

Table 7.4 CD8 immunopositive cells within the graft

Group/Grade	1	2	3	4
Single intrastriatal graft in adult host	++++	++	++	+
P0 striatal graft followed by a contralateral adult graft	++	++	+++	++
Ip + striatal graft into adult (10d ENP)	++++	++++	+++	+
Ip + striatal graft into adult(Primary)	+++++++	+++	++	

Table 7.4 CD8 positive cells within the graft were graded according to Larsson (Larsson et al., 1999b). Immune positive cells were identified in areas of rejection and animals where no surviving graft was evident showed only signs of rejection. (each + represents one animal)

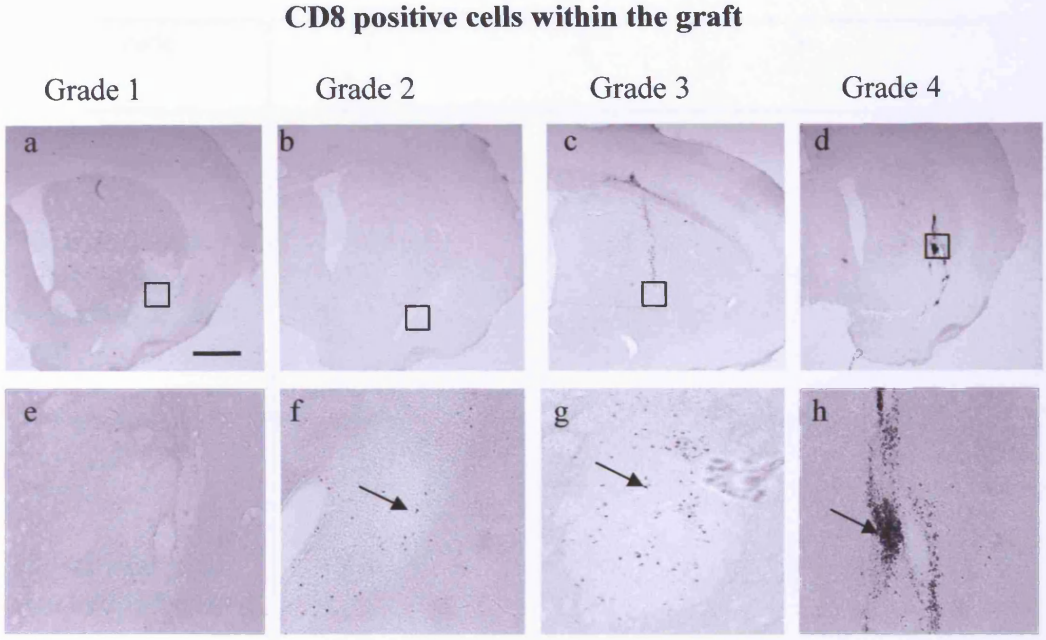


Figure 7.5. The degree of rejection within the graft was measured based on the presence of CD8 positive cells (arrows), graded as described above. With increased rejection there is an increase in the number of CD8 positive cells. Rejected grafts (d and boxed at higher magnification h) show the infiltration of the graft area with CD8 positive cells. In such grafts there were little or no signs of positive surviving donor cells. Scale bar 500μm (boxes are to scale).

Table 7.5 OX42 immunoreactivity within grafts

Group/Grade	1	2	3	4
Single intrastriatal graft in adult host	++++	+++	+	+
P0 striatal graft followed by a contralateral adult graft	+	+	+++	++++
Ip + striatal graft into adult (10d ENP)	++++	++++	++++	+
Ip + striatal graft into adult(Primary)	++++++	+++++	+	

Table 7.5 OX42 positive cells within the graft were graded according to Larsson (Larsson et al., 1999c). Immune positive cells were identified in areas of rejection and animals where no surviving graft was evident showed only signs of rejection. There was a similar trend observed for both CD8 and OX42 immunoreactivity within the grafts. (each + represents one animal)

OX42 immunoreactivity within grafts

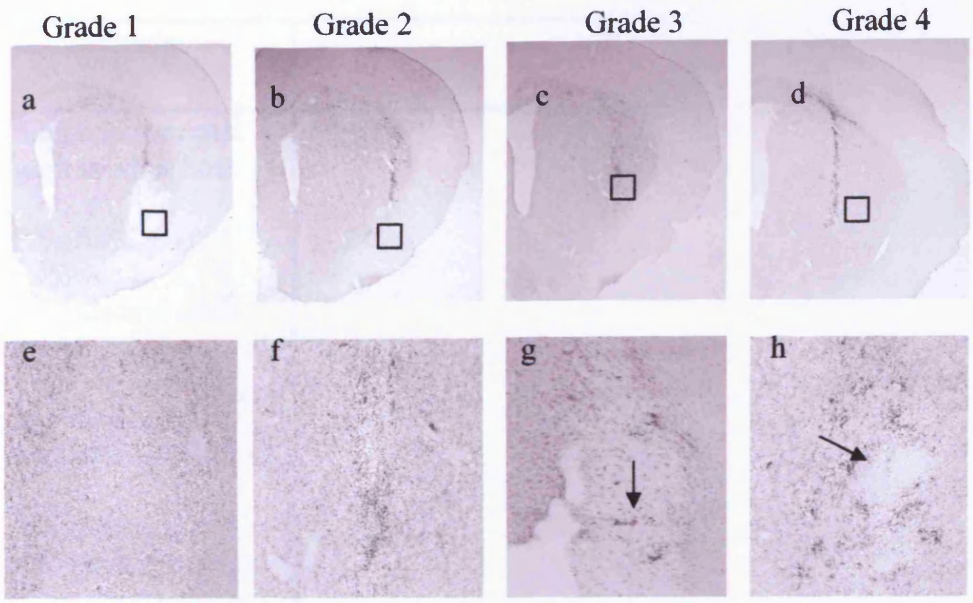


Figure 7.6. The degree of rejection within the graft was also measured based on the presence of OX42 positive microglial cells. OX42 positive cells within the graft were graded as described above. OX42 positive cells were observed along the needle tract in almost all graft and with increased rejection there is an increase in the number of OX42 positive cells in the graft core. Rejected grafts (d and boxed at higher magnification h) show the infiltration of the graft area with OX42 positive cells. In such grafts there were little or no signs of positive surviving cells. Scale bar 500μm (boxes are to scale).

Table 7.6 C3 immunoreactivity within grafts

Group/Grade	1	2	3	4
Single intrastriatal graft in adult host	+ (x9)			
P0 striatal graft followed by a contralateral adult graft	+ (x6)	+	+	+
Ip + striatal graft into adult (10d ENP)	+ (x11)	+		
Ip + striatal graft into adult(Primary)	+ (x12)			

Table 7.6 C3 immunopositive cells within the graft were graded according to Larsson (Larsson et al., 1999d). Immune positive cells were only identified in a small number of animals indicating that the immune response within the graft was cellular rather than humoral. (Number of animals in each group = 9) (each + represents one animal)

C3 immunoreactivity within grafts

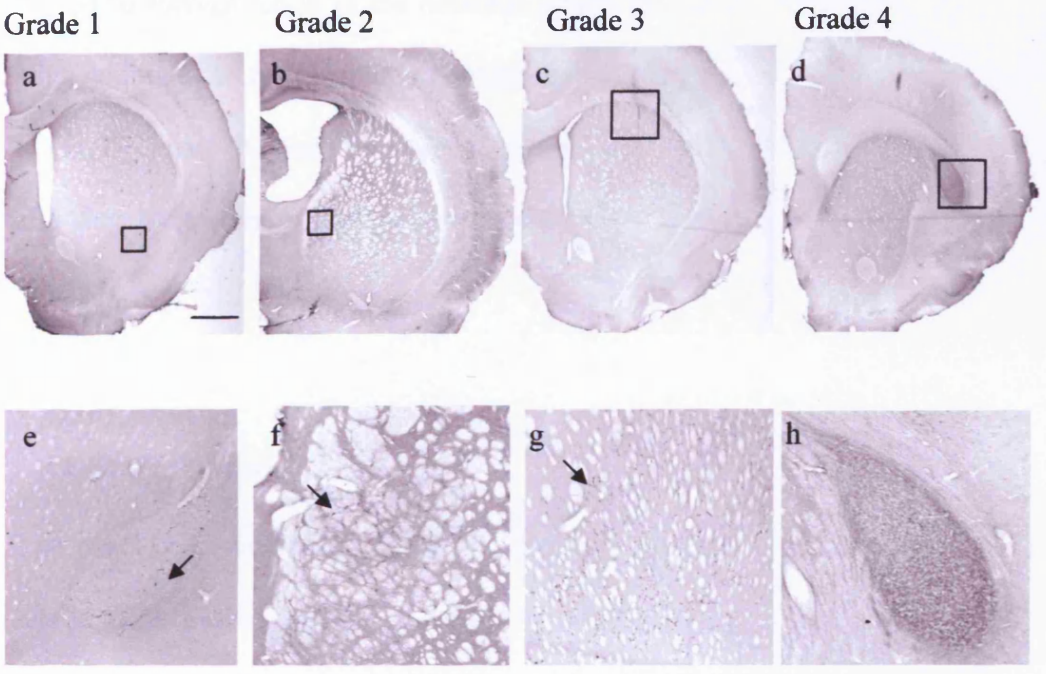


Figure 7.7. The degree of humoral rejection within the graft was also measured based on the presence of C3 positive cells. C3 positive cells within the graft were graded as described above. C3 immunoreactivity within grafts. Little or no immunorectivity was observed in all grafts with only 3 grafts showing immune positive cells.
Scale bar 500μm (boxes are to scale).

7.4 Discussion

Work presented in this chapter provides evidence that it is possible to tolerise an animal to foreign tissue in the neonatal period for subsequent transplantation in the adult without the need for immunosuppressive therapy. This non-invasive method would eliminate the need for ongoing immunosuppression throughout the experiment, allowing long term experiments to explore the potential of human tissue for brain repair.

7.4.1 Graft survival

The histological evidence of ongoing rejection observed in CsA immunosuppressed rats in these grafts suggests that immune mechanisms may be the principal reason for the variable survival in this paradigm. Results clearly demonstrate substantially improved survival following tolerisation of animals in the neonatal period. This was most marked with ip injections of primary tissue and least marked in animals receiving a neonatal intrastriatal graft followed by a contralateral intrastriatal graft in the adult. The improved survival in the animals previously tolerised with primary striatal cells and receiving primary grafts compared to those tolerised and transplanted with ENPs may have an immunological basis; for example, differences between the two populations of cells (primary vs ENPs), as rejection of histocompatible neural transplants has been shown to vary depending on the cellular composition of the grafted material (Bartlett et al., 1990). Another explanation and a more likely one may be due to a compromise of the cell viability following cryopreservation ie ENPs were subject to suboptimal cryopreservation and work is ongoing to improve this technique. This would explain the differences between the data presented here and that reported with pig tissue (Armstrong et al., 2002), i.e. that ENP grafts yielded

better survival than primary grafts; however in the Armstrong study ENPs were not cryopreserved.

One possible explanation for the lower survival of graft tissue seen in animals receiving a neonatal intrastriatal graft followed by a contralateral striatal graft in the adult period may be a result of the repeated attack on the host blood brain barrier as a result of two sequential penetrations of the brain. During the period immediately after graft implantation and before the blood brain barrier reforms, the grafted tissue is more susceptible to immunological rejection and so a pre-existing antibody or activated lymphocytes that may have been generated in response to the first graft could lead to the rejection of the second graft. The good graft survival observed in the neonate only group would lead one to hypothesise that P0 intrastriatal grafts produce a partial tolerance or no tolerance but remains protected from the immune system so that the second penetration of the brain exposes both grafts to immune attack. The small volume of both grafts and the expression of immune rejection markers are supportive of this idea. In some instances, only a scar remained indicating that the supposed immune response was complete. Another possibility is that ENPs injected into the CNS is less effective at tolerising and thus making the brain susceptible to immune attack upon subsequent penetrations or the tolerance may be short lived again making both grafts subject to rejection.

7.4.2 Mechanisms of rejection

Cellular immunity is believed to be important in the rejection of neural xenografts (Low et al., 1999). The markers CD8 and OX42 were used in this experiment to determine the degree to which cellular immune components were recruited to graft

rejection. OX42 recognises activated microglia and cells with a typical microglial morphology were seen surrounding the graft periphery and needle tract of 'healthy' grafts in small numbers, and heavily infiltrating 'rejecting' grafts. These cells have been proposed to represent an afferent limb of the immune response in that they may act to initiate an immune response by presenting xenoantigens to T-lymphocytes (Finsen, 1993b; Geny et al., 1995; Finsen, 1993a; Lund et al., 1994; Poltorak and Freed, 1989a; Armstrong et al., 2001b; Larsson et al., 2001b; Larsson et al., 2000b). Microglial activation has consistently been shown to be an early event following neural transplantation (Mason et al., 1986; Lawrence et al., 1990; Duan et al., 1995; Shinoda et al., 1996) and was evident in this study especially in animals showing overt rejection. The presence of OX42 positive cells in this study thus supports the notion that microglial activation may be an initiating event in xenograft rejection although in this study there was no analysis of graft rejection over time. Another explanation for the presence of OX42 positive cells within grafts, is that they may be involved in carrying out antibody-dependent-cell-mediated cytotoxicity (ADCC) (Fanger et al., 1989). Microglia are also known to act as phagocytes and thus the presence of OX42 positive cells in grafts where only a scar remains may be indicative of such a mechanism (Davis et al., 1994). Thus the role of activated microglia in graft rejection is one that requires further work to elucidate the exact mechanisms of such cells.

CD8 is an accessory molecule expressed on cytotoxic T-lymphocytes, those cells involved in cell killing and it has been shown in rat studies to be ubiquitously expressed on cells of a macrophage lineage and on NK cells (Wallgren et al., 1995a). In this study CD8 positive cells had a morphology that is characteristic of

lymphocytes. There have been several studies that have reported CD8 positivity in rejecting xenografts (Sumitran-Holgersson et al., 2003; Wallgren et al., 1995b; Wennberg et al., 2001; Duan et al., 2001; Barker et al., 2000b; Armstrong et al., 2001b; Larsson et al., 2001b). There are differences of opinion in relation to the importance of these cells in graft rejection. However, it is clear that these cells do play a role in the rejection mechanism although the extent of this role is unknown. In this study there was a clear increase in the expression of CD8 positive cells in rejecting grafts compared to that of 'healthy surviving' grafts suggesting that, along with the OX42 results, there is a cellular immune response ongoing in these grafts.

C3 is a major component of the complement system and is referred to as an opsonin given its ability to bind to neutrophils and macrophages in preparation for phagocytosis. The absence of any significant C3 immunoreactivity within the grafts suggests that the humoral mechanisms were not prominent.

The apparent tolerance in the experiments outlined here requires further work to elucidate the underlying mechanisms. One possibility is that the cells administered intraperitoneally are inducing a macrophage response which then acts via antigen presenting-cells to reduce or eliminate the immune response following intracerebral transplantation. Therefore further experiments will aim to identify what happens to ip injected cells. The differences in graft volumes reported here may be a result of poor survival of cryopreserved cells following thawing resulting in poor cell suspensions on grafting. However in the case of animals receiving bilateral intrastriatal transplants, the reduced graft volume may be a result of an ongoing immune response in the host brain.

Further experiments are required to validate this model system. It has been shown here that cortical primary foetal tissue from different gestational ages and donors could be used to tolerize the animal to human tissue. It would be interesting to further explore the mechanism involved using various tissue types such as muscle or skin tissue as the tolerising tissue and to establish what is happening to the ip injected cells. These experiments are currently ongoing within the lab. There may also be implications for trial design in the ongoing trials of primary foetal tissue (see general discussion).

7.4.3 Conclusion

The model system presented here has potential importance for long term studies of new human donor cells for neural transplantation. It would present an alternative to the use of immunosuppression or immune-incompetent host experimental animals. Also, from the available literature it is evident that the neonatal host, whilst yielding useful information, is not an ideal model system given the differences between it and the adult host environment.

Chapter 8

General Discussion

Neural transplantation can be an effective strategy for treating neurodegenerative disorders such as HD; however, its clinical application is limited by the absence of a suitable and readily-available source of donor neurones. This thesis attempted to assess the potential of embryonic/foetal neural stem cells (ENPs) to provide a source of neurones suitable for transplantation and their ability to reform the damaged circuitry in a lesion model of HD.

8.1 In vitro characteristics of ENPs

ENPs have been shown to have the potential to undergo asymmetrical and symmetrical division over time in culture. There has been a considerable amount of data produced over the last number of years in relation to the culture conditions necessary for the expansion of ENPs. However, there are still a lot of ambiguities in the field and direct comparisons between experiments are difficult given the heterogeneity in protocols used for this purpose. It has been widely accepted that EGF and FGF-2 are important mitogens, albeit not the only mitogens, for the expansion of ENPs in culture, and in the case of human ENPs, LIF is also an important factor. However, despite multiple studies characterising their expansion in number in the presence of these mitogens, there is relatively little known of their effect on the neuronal differentiation of these cultures over time, and this was the

focus of Chapter 3. In this chapter it was found that the concentration of FGF-2 and EGF can influence neuronal differentiation (Kelly et al., 2003; Kelly et al., 2005), and this thus emphasises that it is important to consider the effect of mitogens on the differentiated phenotype as well as the increase in cell number. It would appear that lower concentrations of EGF with a higher concentration of FGF-2 are the preferred proliferation condition for subsequent neuronal differentiation. From this study it is clear that further work is required in this area to understand more clearly the conditions necessary for optimal culture of these cells in relation to their potential use for neural transplantation. This also has relevance to chapter 5 in terms of maximising the potential of the short term expanded cells.

The data arising in Chapter 4 provides an insight into the turnover of neuroblasts within the ENP population over time. In the culture conditions employed in this experiment, a continuous turnover of neuronal precursors was observed (as measured by BrdU incorporation), over the time period studied. It was clear from this data that neurons continue to be born in culture over several passages, although the proportion of neurons (relative to total cell number) declined over time. The precise reason for this proportionate loss of neurons was not clear, although the data pointed to dilution by newly born astrocytes as a major factor. Whether there is an additional loss of neuroblasts or a switch of these progenitors to produce glia, could not be determined from this study and will require further analysis. Recent evidence suggests that the GFAP positive cells that are observed in these cultures may in fact be a renewable multipotential population of cells rather than a true astrocyte (Alvarez-Buylla et al., 2001) and therefore this population of cells may have the potential to produce neurons under the right conditions.

Some other studies in the literature suggest that mouse ENPs may continue to produce neurons over extended periods of time (Smith et al., 2003), and it would be interesting to extend the BrdU studies to look at cultures expanded for much longer periods of time (months and years). Equally, species differences are known to exist (Kelly et al., 2005; Smith et al., 2003; Svendsen et al., 1997b) and, whilst studies in Chapter 3 and Chapter 4 were carried out in mouse tissue, owing to the problematic and limited supply of human tissue in the early parts of this PhD, it is of great importance that the experiments presented here are repeated in human tissue.

A further question that arose from these studies was whether the cumulative neuronal yield was as great as should be expected and whether cells undergoing their last division in the proliferating culture may die in culture and be lost. Whilst a precise answer to this requires further work, it is likely that the current culture conditions are not optimal for the long term survival of these cells, and manipulation of the culture conditions may be necessary for them to survive long term within the population.

The ability to grow these cells in large numbers not only has implications for neural transplantation but is also relevant to an understanding the general biology and the development of human and murine neural tissue, of major influence on such work will be the identification of new markers of striatal differentiation. The identification of gene expression markers is one potential approach. Understanding the role of these genes in striatal development may also be important for directing the differentiation of ENPs to generate striatal-specific neuronal populations.

8.2 In vivo characteristics of ENPs

If ENPs are to be considered for neural transplantation in HD, it is essential, that they are of functional benefit in lesion models of the disease. It has been shown that there is a strong correlation between functional outcome and post mortem histological measures of neurochemical phenotypes within primary grafts. In the excitotoxic lesion model of HD the amount of striatal-like tissue (i.e. DARPP-32 positive tissue) correlates with most measures of behavioural recovery (Fricker et al., 1997b; Fricker et al., 1997a). Therefore, in this thesis, DARPP-32 was used as a reliable measure of striatal differentiation of ENPs both *in vitro* and *in vivo*.

In chapter 5 it was shown that 10 day expanded human striatal ENPs had the potential to differentiate into DARPP-32 positive neurons. There was a decline in the number of neurons differentiating from the culture after 10 days expansion; however a large proportion of these neurons retained the potential to differentiate into DARPP-32 positive neurons. *In vivo*, the DARPP-32 specific differentiation of both primary and ENP grafts was lower than might be expected considering the *in vitro* results. This may indicate that DARPP-32 positive cells survived the transplantation process poorly, although this would be surprising considering studies using rodent fetal striatum as the donor tissue (Wictorin, 1992). Or it may be that human tissues require a prolonged time *in vivo* to differentiate into mature neurons, this is the more likely explanation given that rodent tissue grafts have been shown to develop a mature phenotype 6-8 weeks post-transplantation (Watts et al., 2000c). The puzzle remains as to why DARPP-32 levels are apparently higher *in vitro* after only 7 days differentiation. One possible explanation for this is that the cells differentiating *in*

vitro are mature striatal cells that were present in the primary tissue and the low level seen following transplantation indicate that these mature cells don't survive transplantation well but that less mature precursor cells, that can go on to differentiate into mature neurons, form the major neuronal population in the graft This is supported in a study by Fricker-Gates (Fricker-Gates et al., 2004b) who reported, using BrdU, that at least a proportion of the graft tissue comprised neurons from precursor cells that divided following transplantation. However, the fact that DARPP-32 positive neurons were observed within the graft indicates that ENPs do indeed have the potential to differentiate into the appropriate phenotype in vivo, and that longer term survival studies are warranted.

Another strategy may be to pre-differentiate the cells prior to transplantation. In a PD model it has been shown that pre-differentiating human ENPs derived from the VM resulted in elevated levels of dopamine in the graft region as well as amelioration of behavioural deficits in comparison to non-differentiated ENP grafts (Wang et al., 2004). This method would require careful manipulation of the cells during the transplantation procedure as differentiated neurons may be more susceptible to damage and also the degree to which the cells are differentiated prior to transplantation would need to be optimised. The identification of specific genes associated with striatal development as described above, would provide new striatal-specific markers that are much needed to allow a detailed characterisation of ENPs differentiation both *in vitro* and *in vivo*.

No functional analysis of these grafts was carried out in this study although it is likely that, given the low numbers of mature neurons within the grafts that there would be

no improvement on behavioural analysis at this time point (12 weeks), although long term graft survival may prove functional benefit. As discussed in more detail below, a model system is required to be able to carry out such experiments without the need for ongoing immunosuppression.

8.3 Neuronal projections in vivo

One of the underlying maladies of HD is the damage to the striatal circuitry. Therefore, it is crucial that for a cell therapy to be of benefit in HD, it must be able to reform this lost or damaged circuitry. The potential for ENPs to send out neuronal projections in the host brain was addressed both in the allograft and xenograft environment in this study using both mouse and human tissue to address the xenograft issue. It was found in this study that human derived striatal ENPs had a greater potential to send out long projections into the host brain than did mouse derived striatal ENPs which may be a result of phylogenetic differences between the two species. To confirm this, further studies are required, for example, transplanting fetal pig tissue into a rat host and comparing this to fetal rat tissue transplanted into to pig. Pig ENPs have been grafted to the rat brain and it was found that, like human to rat grafts, pig ENPs were also able to send long projections in the host brain (Armstrong et al., 2003b; Armstrong et al., 2002), however, in this study there was no other graft paradigm employed for comparison.

The specificity of projections is clearly an important issue, and indeed, non-specific projections may have adverse effects. In a study by Armstrong pig ENPs transplanted into a rodent host striatum were reported as producing profuse non-specific projections (Armstrong et al., 2000). In my studies, these non-specific projections

were not seen to the same degree. In fact, the only non-specific fibres were seen traversing the corpus callosum on the ipsi- as well as the contralateral side.

For grafted cells to send out fibre projections in the host brain the neurons must have: (i) the intrinsic ability to do so and (ii) the surrounding environment must be permissive to such growth (Fawcett, 1997; Goldberg and Barres, 2000b). The technique of neural transplantation induces trauma to the host brain with a resulting astrocytosis. This then causes changes in the surface proteoglycan expression and secretion of extracellular matrix, along with damage to the oligodendrocytes (Barker et al., 1996; Chen et al., 2000; Goldberg and Barres, 2000a; Qiu et al., 2000). It is this glial scar that is believed to inhibit the regeneration of axons from the allografted tissue (Dunnett et al., 1989; Brecknell et al., 1996; Bentlage et al., 1999; Wilby et al., 1999). This is in contrast to the studies presented here where projections were seen from allografts albeit to a lesser extent than those observed in the xenograft paradigm. The fact that xenografted tissue is not affected by such inhibitory mechanisms may be a result of differences in growth inhibitory molecules and or receptors thus rendering xenografted tissue unresponsive to such inhibitory cues in the host environment. Alternatively, the protracted development period of human tissue may result in the xenograft tissue being insensitive to the inhibitory envorinment thus allowing human tissue to maintain an embryoninc phase of axon growth for longer periods of time (Fawcett, 1997).

Another factor that may be of importance in relation to assessing the projections from striatal primary and ENP grafts is the ability of the grafted cells to migrate out from the graft following transplantation. In both primary and ENP grafts in this thesis,

migrating cells were identified. Although the numbers were not quantified, it was apparent that there were more migrating cells from ENP than primary grafts. Thus, it is important that migration is taken into account when analysing the projections of striatal grafts. Iontophoresis, which uses an electrical square wave pulse to drive a tracer into the cells, was one method of addressing this issue as the tracer was taken up by graft core only one week prior to perfusion. Differences in the projections from ENP grafts labelled iontophoretically and those labelled using LacZ or GFP suggests that at least some of the additional projections seen in ENP grafts originated from migrated cells rather than from the graft core. Clearly this is important in considering the clinical safety and potential efficacy of ENP grafts, but will also deserve consideration when assessing grafts using donor cells from any stem cell source.

The migration of grafted cells may be a detrimental factor for neural transplantation as it may result in cells acquiring site-specific fates that are not necessarily desirable. Non-specific projections seen in ENP grafts may be a result of migrating cells sending projections to the 'wrong' site. Wang (Wang et al., 2004) suggests that the pre-differentiation of ENPs prior to transplantation may be a way of overcoming the issue of migration, as it was shown in this study that pre-differentiated cells showed little migration in comparison to non-differentiated ENP grafts. The xenograft environment appears to favour the growth of graft neuronal projections in the studies carried out as part of this thesis. However there is of course no allograft arm for the human primary or expanded ENP donor tissue. One way to explore whether the apparent extensive projections from human donor tissue was related to donor brain size would be to perform the 4 way experiment using pig tissue, i.e the pig brain being bigger than the rat or mouse brain and could thus be used to explore the issue

systematically. Whilst in this study, the xenograft environment would appear to favour graft projections in the host brain it is not possible to say conclusively that this is a result of the xenograft environment per se as it may also be influenced by the migration abilities of the grafted cells in the xenograft environment.

8.4 Animal model for long term transplantation studies

One of the main constraints of the studies carried out in this thesis, and one that affects the understanding of the true potential of human derived ENPs for neural transplantation and their progression into the clinical setting, is the lack of long term post transplantation data. Animal studies using human ENPs result in graft rejection if no immunosuppressive therapy is administered. However, immunosuppressive therapies such as daily injections of CsA or using nude rats or scid mice are are limiting, as behavioural analysis is restricted due to the housing environment required for these animals. Therefore, it is important that a model system be developed that allows graft survival and differentiation as well as functional integration to be assessed without the added complication of immunosuppression. Chapter 7 has addressed this issue by considering the ability to tolerise animals to foreign tissue during the first week of the postnatal period, before the immune system has fully developed. Both primary and ENP grafts were analysed following tolerising with the same tissue by ip injection in the neonate, and lead to substantially improved graft survival. Furthermore, there was better graft survival in the case of primary grafts, which is in contrast with the small number of studies so far on this topic, that suggests that ENP grafts are less immunogenic than primary tissue. The reason for this disparity between the tolerisation using primary fetal CNS and ENPs may be because (i) primary tissue is better at inducing tolerance or (ii) cryopreservation of ENPs

compromised their viability. Also it should be noted that following cryopreservation the cells were expanded for 10-14 days before grafting to return the cells to a proliferative state. Therefore, the cells may have undergone changes during this time that may have rendered them significantly different from the cells used to tolerise the host in the neonatal period. Methods for improving the cryopreservation of human tissue are being investigated within the lab.

The results presented in Chapter 7 showing that an intrastriatal graft in the neonatal period followed by an intrastriatal graft on the contralateral side in adulthood resulted in poorer survival of the adult graft than if the tolerisation was performed ip (but better than if no tolerisation took place), could have relevance to ongoing clinical studies: some HD patients have received sequential bilateral human striatal tissue transplants, up to 18 months apart. In one study, one patient, showed initial improvement after the first graft, but deteriorated dramatically after the second graft and an MRI brain scan showed the presence of a cyst like structure at the graft site (Bachoud-Levi et al., 2002b). One could hypothesise that the second graft caused an acute immune response resulting in both grafts being rejected, a concept that would be consistent with an interpretation of our results. This hypothesis will require further studies to elucidate the precise underlying mechanism, and a more appropriate study would be to perform sequential grafts in an adult host animal. Animal studies using allograft tissue, which is less susceptible to immune rejection, has demonstrated that intracerebral grafts in presensitized hosts (animals were sensitized with donor spleen cells prior to intracerebral transplantation) do not survive well (Mason et al., 1986). Widner (Widner and Brundin, 1993) has shown that bilateral allogenic grafts can survive and show improvement on rotational analysis up to 40 weeks post grafting.

However, it was shown that in 5/9 cases the second graft was clearly smaller than the first graft suggesting that there was some rejection ongoing in the second graft. In a second study by the same group (Duan et al., 1993a) there was no trend observed in relation to graft size. However, it was again observed that sequential grafting in the allograft situation into the rodent brain did not affect graft survival. There was an increase in the expression of MHC class II in and around the second graft and this was more pronounced than that seen in animals receiving only one graft. This may be due to the host being immunised by the first graft inducing a low level immune response, although but this moderate increase in MHC class II expression does not correlate with graft rejection (Duan et al., 1993b; Poltorak and Freed, 1989b). Where there is a significant increase in MHC expression there is a correlation with graft rejection (Lawrence et al., 1990; Mason et al., 1986). This work thus raises questions that may be crucial for the way in which transplants are administered in the clinical setting. A requirement to perform one stage bilateral grafts to reduce the risk of rejection would raise the importance of an alternative cell source to provide sufficient quantities of human foetal tissue.

Concluding remarks

This thesis addresses several issues in relation to the use of ENPs as an alternative cell source for transplantation in HD. Transplantation of ENPs into animal models of HD has over the last 5-10 years highlighted a number of problems for the potential clinical use of these cells that are still to be solved, and in recent years the focus has switched back to an emphasis on determining the fundamental characteristics of these cells, although a better understanding of the nature of these cells awaits the identification of more precise cellular markers of precursor differentiation. Moreover, it is clear that

the basic culturing of the cells prior to transplantation is far from optimised and warrants further consideration. It is not clear currently whether long term expanded human ENPs have the capacity to produce sufficient numbers of neurons of the appropriate phenotype to be of clinical use, and these issues may take a substantial amount of time to address satisfactorily. Equally, other stem cells sources also bring with them issues of how to direct the final differentiated phenotype and also safety issues. However, an interim step may be to capitalise on the already-committed cells that can be expanded from ENPs over the first couple of weeks in culture. Even a modest increase in cell number may be advantageous at this early stage in the field, and could provide a starting point for understanding the issues associated with stem cell transplantation. However, it is clear that it would not be appropriate to consider taking such cells into the clinic at this early stage. The next steps are to assess whether these cells can improve behavioural deficits in animal models of HD and to try to improve the rate of proliferation and cell survival within this window of expression.

Caution in this respect is of utmost importance: the recent criticisms of neural transplantation arising from studies carried out in the US (Freed et al., 2001) have already added an air of scepticism to the field, thus it is imperative that we are confident experimentally that any stem cell source is safe and efficacious in animal models before considering pilot human studies.

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Appendix 1

Solutions and Recipes

Culture Media:

Dulbecco's modified eagle's medium and Hams-F12 with 1% PSF (penicillin, streptomycin, fungizone). Either 2%B27 or 1%N2 was added to this medium along with the appropriate concentrations of EGF and FGF-2.

Differentiation media:

The basic medium of DMEM plus 1% PSF as well as 2%B27 and 1% fetal calf serum (FCS).

Freezing media:

7% DMSO (Dimethyl-sulphoxide) and 15% BSA in basal medium. For 33mls, 30.5ml basal medium, 5g BSA and 2.5ml DMSO are used.

Trypsin solution:

Mix 0.1% trypsin with 0.05% DNase in $HBSS^{--}$.

DNase solution:

Mix 0.05% DNase into dissection medium.

Phosphate Buffered Saline:

Constituents include 8.5g sodum chloride, 0.4g dihyrogen sodium phosphate and 1g disodium hydrogen phosphate per litre of distilled water. The pH was 7.4, adjusted using hydrochloric acid.

Quench:

Made up with 10ml concentration hydrogen peroxide, 10ml methanol and 80ml distilled water, reduces background staining.

TRIS-buffered saline:

This is made up as a 4x stock solution. 48g of Trizma base and 36g of sodium chloride were added to 1 litre of distilled water. The pH was adjusted to 7.4 using hydrochloric acid.

To make TXTBS 0.2% Triton-X100 was added to the solution, this allows membrane permeabilisation.

For the long term storage of tissue sections, the addition of 0.02% sodium azide is added to the stock solution, this prevents any microbial growth from taking place.

TRIS non-saline solution (0.05M Trizma solution):

6g of Trizma base is added to 1 litre of distilled water and again the pH is adjusted to 7.4 using hydrochloric acid.

Perfusion buffer:

18g of di-sodium hydrogen phosphate and 9g sodium chloride were added to 1 lire of distilled water. Orthophosphoric acid was used to adjust the pH to 7.4.

4% Paraformaldehyde:

To 1 litre of perfusion buffer 40g of paraformaldehyde is added. 10M sodium hydroxide was added over heat to help dissolve the paraformaldehyde. Orthophosphoric acid was used to adjust the pH to 7.4.

25% Sucrose solution:

250g of sucrose is made up to 1 litre using 750ml perfusion buffer.

Cresyl fast Violet Protocol (nissl stain):

Cresyl violet is made up by dissolving 5g cresyl violet in 600ml of distilled water, and then adding 600ml of 1M sodium acetate and 340ml of acetic acid, folowed by stirring and filtering. A Shandon processing machine is used for staining.

Distilled water	3x5 min
70% alcohol	5 min
95% alcohol	5 min
100% alcohol	5 min
50/50 chloroform/alcohol	20 min
95% alcohol	5 min
70% alcohol	5 min
Distilled water	5 min
Cresyl violet	5 min
Distilled water	end

Sections are then cleared and dehydrated on removal from the machine:

70% alcohol	5 min
95% alcohol	5 min
Acid alcohol	5 min
100% alcohol	5 min
Xylene	5 min

Slides are then converslipped using a DPX mountant.

Appendix 2

Materials & Suppliers

b-FGF	R & D Systems	EGF	Sigma
DMEM-F/12	GIBCO	HBSS$^-$ Solution:	GIBCO
DMSO	Sigma	BSA	Sigma
B27	GIBCO	Hepes buffer	GIBCO
PSF	GIBCO	L-Glutamine	GIBCO
FCS	GIBCO	NGS	Dako
Isoflurane	Vericore	Dnase	Sigma
Trypan blue	Sigma	O_2	BOC Gases
Euthatal	Rhône Merieux	N_2O	BOC Gases
Glucose	Sigma		
Trypsin	Worthington Biochemical Corporation		

Molecular Probes	Leiden, Netherlands
Chemicon	Southampton, UK
Sigma	Poole, Dorset, UK
GIBCO	Paisley, Scotland
R & D Systems	Abingdon, Oxon, UK
Worthington Biochemical Corporation	Freehold, New Jersey, USA
Vericore	Marlow, Buckinghamshire, UK
Rhône Merieux	Harlow, Essex, UK
BOC Gases	Manchester, UK
Dako	Glostrup,Denmark

Appendix 3

Antibodies for immunohistochemistry

IMMUNO-HISTOCHEMISTRY- Primary, Secondary & Blocking Sera													
PRIMARY Antibody	Raised in	Supplier	Dil'n	ul per ml	Normal Serum	Blocking Dil'n	ul per ml	'Normal' Dil'n	ul per ml	SECONDARY Antibody	Dil'n	ul per ml	Comments
B Galactosidase	**Rabbit**	Cappel	1:6000	0.16	**Goat**	3%	30	1%	10	**Goat** anti-rabbit	1:200	5	
BIII Tubulin	**Mouse**	Sigma	1:400	2.5	**Horse**	3%	30	1%	10	**Horse** anti-mouse	1:200	5	*2ndry Rat adsorbed*
BrdU	**Mouse**	Roche	1:25	40.0	**Horse**	3%	30	1%	10	**Horse** anti-mouse	1:200	5	*2ndry Rat adsorbed*
CALBINDIN	**Mouse**	Sigma	1:20,000	0.05	**Horse**	3%	30	1%	10	**Horse** anti-mouse	1:200	5	*2ndry Rat adsorbed*
CD8	**Mouse**	Serotec	1:500	2.0	**Horse**	3%	30	1%	10	**Horse** anti-mouse	1:200	5	*2ndry Rat adsorbed*
ChAT	**Rabbit**	Chemicon	1:2,000	0.50	**Goat**	3%	30	1%	10	**Goat** anti-rabbit	1:200	5	
DARPP-32	**Mouse**	Cornell Univ	1:30,000	0.03	**Horse**	3%	30	1%	10	**Horse** anti-mouse	1:200	5	*already diluted 1:50 ∴ Needs 1:600 diln.*
GFAP	**Rabbit**	DAKO	1:2,000	0.50	**Goat**	3%	30	1%	10	**Goat** anti-rabbit	1:200	5	
GFP	**Chicken**	Chemicon	1:4000	0.25	**Goat**	3%	30	1%	10	**Goat** anti-chicken	1:200	5	
HuNu	**Mouse**	Chemicon	1:1500	0.67	**Horse**	3%	30	1%	10	**Horse** anti-mouse	1:200	5	*2ndry Rat adsorbed*
M2	**Rat**	Bjorklund Lab	1:50	20.0	**Rabbit**	10%	100	5%	50	**Rabbit** anti-rat	1:200	5	*}No Triton Use 10% in }primary then*
M6	**Rat**	Bjorklund Lab	1:50	20.0	**Rabbit**	10%	100	5%	50	**Rabbit** anti-rat	1:200	5	*}5% for 2ndry(ABC 1%)*
NeuN	**Mouse**	Chemicon	1:4,000	0.25	**Horse**	3%	30	1%	10	**Horse** anti-mouse	1:200	5	*2ndry Rat adsorbed*
OX-42	**Mouse**	Serotec	1:500	2.0	**Horse**	3%	30	1%	10	**Horse** anti-mouse	1:200	5	*2ndry Rat adsorbed*
Parvalbumin	**Mouse**	Sigma	1:4,000	0.25	**Horse**	3%	30	1%	10	**Horse** anti-mouse	1:200	5	*2ndry Rat adsorbed*
Tau	**Mouse**	Zymed	?1:100	10.0	**Horse**	3%	30	1%	10	**Horse** anti-mouse	1:200	5	*2ndry Rat adsorbed*

Appendix 4

GFP expression over time in vivo

GFP expression over time *in vivo* was analysed. Primary and 10 day expanded grafts were administered (4 animals per group for each time point). Grafted animals all received the same number of cells and were sacrificed at 3, 6, 12 and 20 weeks post transplantation. It was found that by 12 weeks the expression of GFP within the grafts was significantly diminished and by 20 weeks there were no GFP positive cells in any of the grafted animals. 3 and 6 week grafts were similar to those described in Chapter 5, however, by 12 weeks the graft volume as measured by GFP positive cells had decreased and by 20 weeks there were only occasional GFP positive cells identified.

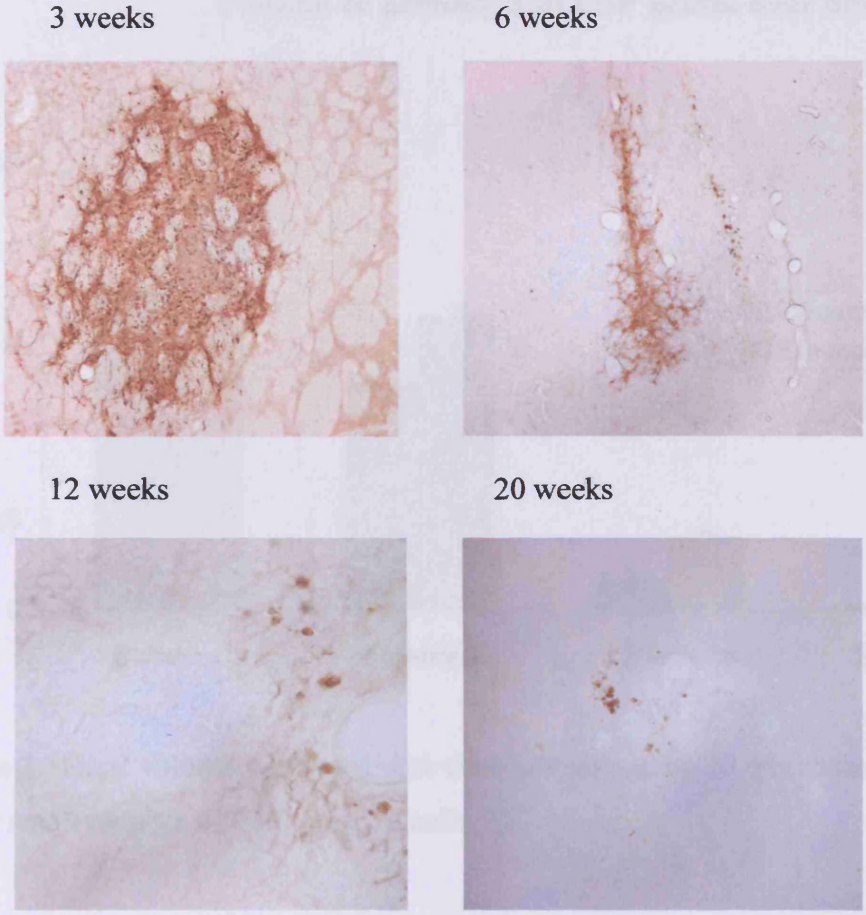

Figure 1. Graft morphology at the various time points analysed in this study.

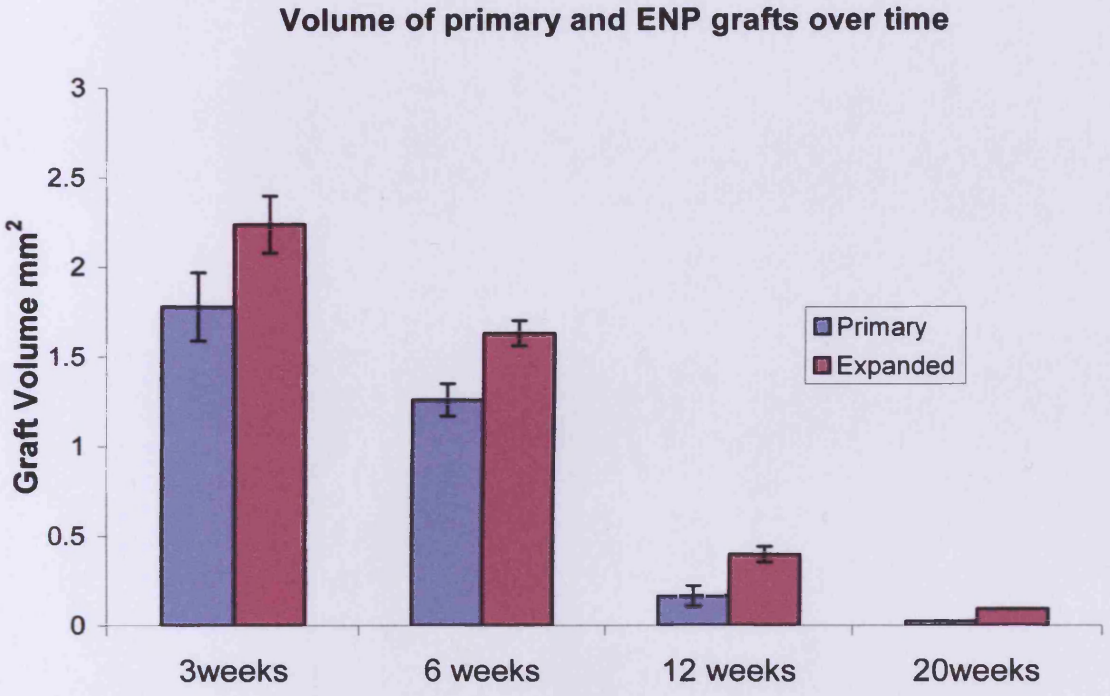

Figure 2. Graft volume decreased with time in vivo and by 20 weeks there were only a very small number of GFP positive cells.