Validation and characterisation of a new method for *in vivo* assessment of human donor cells

This dissertation is submitted for the degree of Doctor of Philosophy at Cardiff University

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Summary

This thesis encompasses a range of experiments designed to characterise and validate a method of desensitising rodent hosts in the neonatal period to human tissue in order to promote the survival of human striatal grafts in the adult host. The successful application of this method is important to allow the preclinical testing of potential human donor cells for therapeutic transplantation, specifically in neurological disease. Demonstrating safety and functionality of transplanted human cells in rodent hosts requires long term assessment of surviving grafts, for which current immune suppression methods are insufficient. These experiments were therefore designed to determine the optimum parameters of a previously described method of desensitising rats to human tissue and to validate this method in mice.

In order to determine whether the same type of human donor tissue must be used to inject neonatally as will be used for the later transplant; **Chapter 3** compares survival of transplants of human neural tissue into the striatum of rats desensitised with a variety of human tissues. It was found that it is not necessary to use human neural tissue to desensitise hosts to human neural transplants and data is suggestive of improved survival using liver cells to desensitise as measured by graft survival and the host immunological response

The aim of **Chapter 4** was to investigate whether desensitisation in the neonatal period is specific to the species of tissue used, or whether a reduction in immune response to all subsequent transplants has been induced. Animals were desensitised and transplanted with tissue of either matching or mismatching combinations of human and mouse neural cells, including bilateral transplants using both species. Although no survival of mouse transplants was found in any condition, all human transplants were found to survive in hosts desensitised with human tissue and treated with cyclosporine A (CsA) demonstrating successful desensitisation to human tissue. Additionally, half of the human transplants in hosts desensitised with mouse tissue survived, raising questions about the possibility of common epitopes on human and mouse tissue used to desensitise.

Chapter 5 includes a number of experiments designed to determine whether mouse hosts could be desensitised neonatally to human neural tissue. Desensitisation was not found to be successful, however at least half of control hosts treated with CsA were also found to reject human transplants. The findings in this chapter suggest differences in the mouse as a host for human xenografts as compared to the rat. Additionally control animals in these experiments which received striatal grafts of mWGE, showed higher than expected rates of rejection. Therefore **Chapter 6** reconsiders mouse tissue transplantation protocols, testing different donor ages and cell preparations to improve transplant survival. Good survival was found in all preparations in this experiment, potentially related to one of the modifications to transplant protocols, and the use of younger donor tissue was found to produce larger transplants.

These findings provide further support for the neonatal desensitisation method in rat hosts, and suggest the potential for use of non-neural tissue types for desensitisation of neonates. The data presented in this thesis also has implications for the mechanisms underlying the success of the method in the rat. However interpretation of initial mouse experiments was difficult as graft survival was generally poor and even mouse to mouse transplants did not survive to the level expected. Thus this highlights the need to reassess standard immunosuppression protocols in mice, and determine what differs between the rat and mouse rejection response to xenografts.

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Abbreviations

6-OHDA	6-hydroxydopamine
α-Gal	α-galactosyl
ALS	amytrophic lateral sclerosis
AIRE	Autoimmune regulator
APC	Antigen presenting cell
ANOVA	Analysis of variance
aNSCs	Adult neural stem cells
B6	C56BL/6J (mouse)
BAC	Bacterial artificial chromosome
BLI	Bioluminescence imaging
BBB	Blood brain barrier
CEC	Cortical epithelial cell
CNS	Central nervous system
cTEC	Cortical thymic epithelial cell
CS	Cell suspension
CsA	Cyclosporine A
CSF	Cerebrospinal fluid
CRL	Crown rump length
CTL	Cytotoxic T lymphocytes
CTX	Cortex (h = human, m = murine)
CV	Cresyl violet
DAB	Diaminobenzidine
DAG	Diaglycerol
DARPP-32	Dopamine and adenosine 3’5’-monophosphate regulated phosphoprotein.
DC	Dendritic cell
D(hCTX)	Desensitised neonatally (hCTX)
DMEM	Dulbecco’s Modified Eagle’s Medium
DN	Double negative
DNase	Deoxyribonuclease
DP	Double positive

E	Embryonic day
EGF	Epithelial growth factor
ESC	Embryonic stem cell (h = human, m = murine)
eTAC	Extrathymic Aire expressing cell
FACS	Fluorescence activated cell sorting
FGF-2	Fibroblast growth factor
FKBP-12	FK binding protein 12
FNP	Foetal neural precursors
GABA	γ-aminobutyric acid
GE	Ganglionic eminence
GMP	Good manufacturing practice
HBSS	Hanks buffered saline solution
HD	Huntington's disease
Hdh	Wild type murine huntingtin
HDRC	Huntington's disease collaborative research group
hFNPCs	Human foetal neural precursor cells
hGRP	Human glial restricted precursor cells
HLA	Human leukocyte antigen
hPF	Human primary foetal (tissue)
HSC	Hematopoietic stem cell
HTA	Human Tissue Authority
HTT	Huntingtin gene
Htt	Huntingtin protein
HUCB-NSC	human cord blood derived neural stem cells
HuNu	Human nuclear antigen (antibody)
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
iN	Induced neuron
i.p.	Intraperitoneal
iPSC	Induced pluripotent stem cell (h = human, m = murine)
iPSC-NPCs	Induced pluripotent stem cell derived neural precursor cells
KO	Knockout
L-Dopa	Levodopa

MAO-B	Monoamine oxidase-B
MAP	Mitogen-activated protein
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
mTEC	Medullary thymic epithelial cell
mTOR	Mammalian target of rapamycin
MS	Multiple sclerosis
MSC	Mesenchymal stem cell
MSN	Medium spiny neuron
NeuN	Mature neuronal marker (antibody)
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa B
NK	Natural killer (cells)
NOD	Nonobese diabetic
NPC	Neural precursor cell
NSC	Neural stem cell
P	Postnatal day
PD	Parkinson's disease
PDI	Peripheral decarboxylase inhibitor
PERV	Porcine endogenous retrovirus
PF	Primary foetal (tissue)
PFA	Paraformaldehyde
QA	Quinolinic acid
RAPA	Rapamycin (sirolimus)
RGC	Retinal ganglion cells
SCID	Severe combined immunodeficient
SD	Sprague Dawley (rat)
SNc	Substantia nigra (pars compacta)
SWIFT	South Wales Initiative for Foetal Transplantation
TAC	Tacrolimus (FK506)
TBS	TRIS buffered saline
TBZ	TRIS buffered saline and 0.01% sodium azide
TCR	T cell receptor
TEC	Thymic epithelial cell

Tg	Transgenic
TGF-β	Transforming growth factor-beta
TIM	T cell immunoglobulin domain and mucin domain
TH	T helper
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TNS	TRIS non-saline
ToP	Termination of pregnancy (m = medical; s = surgical)
TRA	Tissue restricted self antigens
Treg	Regulatory T cell
TRIS	Trizma base
UHDRS	Unified Huntington's Disease Rating Scale
VCAM-1	Vascular cell adhesion molecule-1
VM	Ventral mesencephalon
WGE	Whole ganglionic eminence (h = human, m = murine)

Contents

Chapter 1

Introduction

Cell replacement offers a therapeutic option for the treatment of a number of diseases of the central nervous system. Successful transplants of human primary foetal tissue in the clinic have been achieved in Huntington's disease, with current work aiming to identify and characterise alternative potential human donor cells for transplantation to avoid ethical and logistical issues with the use of this tissue. Current research in regenerative medicine has offered a number of human progenitor cell types with the potential for use in this area, all of which require preclinical testing through xenotransplantation in animal models of disease. This necessitates immunosuppressive treatments to promote survival of donor cells for sufficient time to assess safety and efficacy of transplanted cells. Immunosuppression strategies available at present do not offer optimum parameters for the adequate assessment of the function of these human donor cells, therefore alternative methods are essential to progress this field of research. The work presented in this thesis includes characterisation and further validation of a method that has the potential to promote long term survival of human donor cells in rodent disease models, thus allowing full functional assessment of a transplant in a lesion model of Huntington's disease. The neonatal desensitisation method discussed is referred to as "tolerising", although with no assumptions of inducing true immunological tolerance.

1.1 Cell transplantation in neurodegenerative disease

The ongoing investigations into neural cell replacement therapy provide a therapeutic strategy for a range of neurological disorders for which currently only limited or symptomatic treatments are available, and where there is an unmet clinical need for alternative effective interventions. Although drug treatments have the potential to alleviate symptoms, currently available pharmacological agents cannot repair damage sustained to the central nervous system (CNS). However, cell transplantation has the potential to replace cells lost due to the disease. Much research into cell replacement therapy has focused on conditions such as Parkinson's disease (PD) and Huntington's disease (HD), as the pathology of both comprises a relatively focused area of primary cell loss thus providing a target cell population to replace. This section reviews the cell transplantation literature in both of these diseases, but with an emphasis on HD, since the work presented in this thesis focuses on rodent HD lesion models. Although proof-of-principle has been demonstrated for the potential efficacy of human (h) primary foetal (PF) tissue transplantation, alternative tissue sources are required for effective translation of this therapy to the clinic.

1.1.1 Parkinson's disease

PD is a common progressive neurodegenerative condition affecting approximately 1% to 2% of the population aged over 60. In Europe alone, recent estimates place the number at 1.2 million people (Gustavsson *et al.* 2011). The aetiology of PD is unclear although it is considered to be attributable to a combination of genetic and environmental factors. Known genetic causes have been identified in only about 10% cases and mainly in early onset PD (Alcalay *et al.* 2010). Classical clinical features of PD include bradykinesia, rigidity and rest tremor progressing to gait disturbances and postural instability. Other non-motor impairments frequently develop including depression and dementia, laryngeal dysfunction and dysphagia, autonomic and sensory disturbances and the condition eventually progresses to cause significant disability with markedly impaired quality of life. The two main neuropathologic findings in PD are loss of the pigmented dopaminergic neurons in the substantia nigra pars compacta (SNc) which project to

the striatum, leading to a reduction in striatal dopamine levels (Olanow *et al.* 1996) and the presence of Lewy bodies at autopsy.

The loss of dopamine is responsible for most of the characteristic motor symptoms observed in PD. Treatment therefore aims to alleviate this dopamine depletion. Currently the main pharmacological agents for treatment of PD include the dopamine precursor levodopa (L-Dopa); usually administered in combination with carbidopa, a peripheral decarboxylase inhibitor (PDI), dopamine agonists and monoamine oxidase-B (MAO-B) inhibitors. L-Dopa enters the remaining dopaminergic neurons where it is metabolised to dopamine, replacing the depleted endogenous neurotransmitter. Dopamine agonists exert their anti-parkinsonian effects by acting directly on dopamine receptors and mimicking the endogenous neurotransmitter. MAO-B inhibitors inhibit the activity of MAO-B oxidases that are responsible for inactivating dopamine. Symptomatic pharmacotherapy is successful to an extent, usually providing good control of motor signs for a number of years. However long term treatment, particularly with L-Dopa, results in the gradual increase in dyskinesias amongst other side effects and fluctuating motor responses with narrowing of the therapeutic window (Brooks 2000). Surgical procedures are reserved for patients with disabling motor symptoms uncontrolled by medication. Currently there is no proven neuroprotective or disease modifying treatment although a variety of agents are under investigation including the MAO-B inhibitors selegeline and rasagiline, which may possibly modify the outcome of PD (Lew 2011), and gene therapy that may aid in the restoration of the nigrostriatal dopaminergic network (Coune *et al.* 2012).

To determine the suitability of cell replacement for treatment in neurological disease necessitates the use of animal models in order to demonstrate safety and efficacy pre-clinically. Neurotoxin lesion models aim to mimic the degeneration seen in disease by inducing the loss of a specific cellular population. In animal lesion models of PD, 6-hyroxydopamine (6-OHDA) is injected and is selectively taken up by dopaminergic neurons in the substantia nigra which subsequently die. Successful neural cell transplants of human primary foetal tissue from the developing ventral mesencephalon (VM) tissue into 6-OHDA lesioned animals have been demonstrated, showing functional integration of grafts (Björklund and Stenevi 1979; Dunnett *et al.*

1981) and improvements on behavioural measures designed to assess motor deficits associated with the PD model (Brundin *et al.* 1986; Brundin *et al.* 1988). Whilst there are limitations with animal models in that the full spectrum of PD symptoms and disease progression is not replicated, these findings in experimental animals have led to numerous clinical trials in PD patients using hPF tissue.

Early open clinical trials in small numbers of patients have produced a number of positive results with transplants successfully restoring dopamine levels in the striatum leading to functional improvements on measures of PD symptoms (Defer *et al.* 1996; Lindvall and Björklund 2004). Patients have shown reductions in Parkinsonian symptoms without additional L-Dopa treatment for a number of years after transplantation (Dunnett *et al.* 2001; Lindvall *et al.* 1990). Long term functional efficacy of grafts in the reduction of motor symptoms has since been demonstrated up to 16 years post-transplantation (Piccini *et al.* 1999), although with continuing decline in non-motor symptoms (Politis *et al.* 2012). Despite these findings, some negative outcomes have had a detrimental effect on the field. Later double-blind controlled clinical trials (including sham operations) in larger numbers of PD patients to evaluate the transplantation of human primary foetal VM, did not provide overall evidence of efficacy, and were reported to result in the emergence of dyskinetic side effects persisting after the withdrawal of L-dopa (Freed *et al.* 2001; Olanow *et al.* 2003). These data seemed to publicly represent negative outcomes of hPF transplants in PD, although some improvements were noted in patients with less severe disease (Olanow *et al.* 2003) and in younger aged patients (Freed *et al.* 2001). Whilst highlighting detrimental side effects for further investigation, these findings were subsequently not considered to be representative of the field since non-standard transplant techniques and assessments were used. The studies were also underpowered, with only short term follow up reported. Subsequent longer term follow up of patients from the Freed trial showed clinical improvement and graft viability sustained for up to 4 years after transplantation (Ma *et al.* 2010). The findings and experience from the earlier studies have led to initiation of the EU-sponsored TRANSEURO project which aims to develop a better cell therapy approach for PD patients using PF dopaminergic cells and an initial open trial of hPF VM transplants in PD patients is now ongoing (Evans *et al.* 2012).

1.1.2 Huntington's Disease

HD is a genetic autosomal dominant neurodegenerative disorder with full penetrance. It is estimated to affect about 5 to 7 people per 100,000 in Western countries, although there is some evidence to suggest the prevalence may be significantly greater (Evans *et al.* 2013; Spinney 2010). Onset of the disorder is insidious with symptoms usually becoming manifest in midlife although it has been reported as starting in infancy and as old as in the 90s. The characteristic clinical features of HD include chronic motor, cognitive and behavioural changes that progress over 15-30 years resulting in profound disability (Novak and Tabrizi 2010). Motor symptoms include chorea, dystonia, bradykinesia, rigidity, postural instability, dysarthria, dysphagia, abnormal eye movements, tics and myoclonus. Cognitive symptoms include deterioration of executive function, short term memory problems and dementia. Psychiatric symptoms associated with HD include a high incidence of depression as a direct result of the disease (Paulsen *et al.* 2005b) with links to increased suicide risk in patients (Paulsen *et al.* 2005a). Patients with HD can also develop psychosis, obsessive compulsive symptoms, sexual and sleep disorders and personality changes. Most patients survive for 10 to 30 years after onset of HD with death being usually from intercurrent illness commonly pneumonia and cardiovascular disease (Sørensen and Fenger 1992).

HD is caused by expansion of a polymorphic CAG trinucleotide repeat encoding of a polyglutamine tract within the Huntingtin gene (*HTT*) on chromosome 4, encoding for the huntingtin protein (htt), resulting in a mutant form of the protein. The genetic defect responsible for HD was mapped to chromosome 4 in 1983 and first identified in a landmark study by the Huntington's Disease Collaborative Research Group 10 years later (HDCRG 1993). The htt protein is essential for neural development although its function is not fully elucidated (Novak and Tabrizi 2010). In the normal population, *HTT* has between 10-29 repeats (Kumar *et al.* 2010). Greater repeat number is considered abnormal, with HD patients having between 36-121, and an inverse relationship exists between repeat length and age of onset on a population basis, with higher repeat length associated with a younger age of onset and greater severity (Ferrante 2009; Kumar *et al.* 2010). The underlying pathology of the disease is characterised by progressive neurodegeneration within

the CNS with prominent cell loss and atrophy in the caudate and putamen. In particular, the γ-aminobutyric acid (GABA)-ergic medium spiny projection neurons of the striatum are lost, with more involvement of the enkephalin containing medium spiny neurons (MSNs) that project to the external globus pallidum, than neurons that contain substance P and project to the internal globus pallidum. Because of preferential involvement of the indirect pathway of basal ganglia-thalamocortical circuitry this contributes to the characteristic chorea observed early in the course of HD. Other affected areas include the substantia nigra, cortical layers 3, 5, and 6, the CA1 region of the hippocampus, the angular gyrus in the parietal lobe, Purkinje cells of the cerebellum, lateral tuberal nuclei of the hypothalamus, and the centromedial-parafascicular complex of the thalamus (Walker 2007). Another pathological feature is the presence in the brain of intranuclear inclusions including amongst other things mutant huntingtin. Whilst initially these inclusions were considered to be toxic, more recently evidence from animal studies suggests that these inclusions may not be predictors of disease activity (Walker 2007). The mechanisms whereby this mutant gene produces cellular dysfunction are as yet not clear. As the mutant HD gene results from an expanded CAG repeat leading to a polyglutamine strand of variable length at the N-terminus it is suggested that this tail confers a toxic gain of function (Walker 2007).

The disease is devastating to both patients and their families and currently there is no treatment available for HD that can prevent or slow progression of the disease. Treatments are aimed at management of symptoms and improvement of quality of life for patients. This may include physiotherapy to improve gait and balance, speech and language therapy, and a number of pharmacological agents that include dopamine-depleting agents such as tetrabenazene to treat chorea, anticonvulsants, antipsychotics and antidepressants (reviewed in Novak and Tabrizi (2010) and Novak and Tabrizi (2011)).

Ultimately targeting the pathology/genetics of the disease is likely to develop optimum treatments. Currently, cell replacement therapy presents an alternative strategy for treatment of HD sufferers, with the potential to delay disease progression and alleviate symptoms. Following on from transplantation of dopaminergic cells in PD; HD was considered to be the next target for transplantation in the early 1990s

(Rosser and Bachoud-Lévi 2012). Delivery of developing striatal neurons from the foetal ganglionic eminence (GE) is targeted homotopically to the striatum with the aim of replacing striatal projection neurons and rebuilding lost connections. Transplantation in rats has shown that rat foetal striatal neurons survive and can integrate and differentiate into functional MSNs with the formation of relevant connections in the host brain (Dunnett *et al.* 2000; Isacson *et al.* 1984; Wictorin 1992). Transplantation studies have been carried out in lesion models of HD, designed to mimic the cell loss seen in patients with the disease. One such model is an excitotoxic lesion model whereby quinolinic acid (QA) is injected into the striatum, targeting the death of MSNs (Beal *et al.* 1986). The injection of QA into the striatum damages GABA-ergic and substance-P containing neurons (typical of MSNs), and spares those known to also be spared in the clinical condition of HD (Ferrante 2009). This provides a valuable model with relevant cell loss in which to study HD pathogenesis and test potential therapeutic strategies. Transplantation of PF striatal tissue into rodents has also been successful in the reconstruction of disrupted circuitry in the QA lesion model, with grafts containing mature striatal neurons and forming relevant afferent and efferent connections in the host brain (Nakao and Itakura 2000; Nakao *et al.* 1999; Wictorin 1992). Additionally, improvements have been observed in a range of motor and cognitive behavioural tasks following PF transplants to the lesioned rat striatum (Dunnett *et al.* 2000; Nakao and Itakura 2000).

A number of genetic models of HD have now been developed; the majority in mice (Ferrante 2009), with the recent production of a transgenic (Tg) rat model (von Hörsten *et al.* 2003). Three main types of mouse models have been generated with varying phenotypes (Ferrante 2009). First; mice expressing exon 1, or 1 and 2 of human *HTT* with varying CAG repeat lengths in addition to both alleles of murine wild type huntingtin (*Hdh*). These include the R6/2 mouse model, the first Tg model of HD to be generated (Mangiarini *et al.* 1996). Secondly; knock-in mice with the insertion of pathogenic CAG repeats into the existing CAG expansion of murine *Hdh,* such as the *Hdh*$^{(CAG)150}$ (Heng *et al.* 2007; Lin *et al.* 2001). Finally mice expressing the full-length human HD gene, with varying CAG repeats (Hodgson *et al.* 1999; Hodgson *et al.* 1996; Reddy *et al.* 1998). Although a number of HD mouse models exist, as yet little successful transplantation has been carried out in these

hosts. Since mouse models of HD express much longer repeat lengths than those found in patients, they develop a much more widespread pathology, more similar to juvenile HD and due to this lack of specific cell loss do not provide ideal models for transplantation. Some transplant studies have been carried out in mouse models; striatal grafts of wild type mouse tissue transplanted into the R6/2 mouse model of HD have been found to survive and produce marginal behavioural improvements 6 weeks after transplantation (Dunnett *et al.* 1998). A more recent study, however, found that transplantation of immortalised human striatal stem cells into the same host yielded very small grafts and no behavioural improvement (El-Akabawy *et al.* 2012). This may be due to issues with survival of xenogeneic human tissue in the mouse hosts.

The recent development of rat models of HD; including a Tg model (TgHD rat - von Hörsten *et al.* (2003)) and bacterial artificial chromosome (BAC)-HD model (Yu-Taeger *et al.* 2012) provide perhaps more appealing hosts, since the majority of transplants and functional improvements have been developed in rats. The TgHD rat model carries expansions of 51 CAG repeats under the endogenous rat HD promoter and has been reported as displaying a phenotype typical of late onset HD, as compared to the majority of mouse models with large CAG repeats, which show juvenile onset and rapid degeneration. The rat model is therefore suggested to be more true to the clinical presentation of HD (von Hörsten *et al.* 2003). However heterozygous TgHD rats display only subtle behavioural deficits, therefore requiring breeding of homozygous animals for testing (Brooks *et al.* 2009). The BAC-HD model contains the full length human *HTT* gene with 97 CAG repeats, exhibiting a stronger phenotype than the transgenic, with earlier onset and faster progression of motor deficits (Yu-Taeger *et al.* 2012). However, these models are relatively new and much work remains for their characterisation. Therefore lesion models still continue to be useful in transplantation studies, providing relevant cell loss and identifiable motor deficits to target replacement and to assess the safety and function of donor cells.

The successful transplantation of rat PF striatal tissue in a number of pre-clinical trials in rat led to further successful trials in non-human primate models (See Nakao and Itakura (2000) for a review). Foetal striatal allografts in excitotoxic lesion

models in primates produced comparable data to rodent studies, with survival, differentiation and integration of grafts leading to improvements on both motor and cognitive tasks (Kendall *et al.* 1998; Palfi *et al.* 1998). Following these findings a number of clinical trials were initiated in HD patients, with the transplantation of hPF tissue. Due to ethical issues surrounding the use of double-blind trials and sham surgery in patients, examined in particular in reference to clinical trials in PD (Dekkers and Boer 2001; Macklin 1999), clinical trials of hPF tissue transplantation in HD patients have so far all been small, open-label trials with a focus on core assessment protocols, as emphasised for PD (Boer and Widner 2002). Initial safety studies showed no acceleration of disease progression or serious, irreversible side effects as a direct result of the transplants, though little clinical improvement was seen in many of the studies (Hauser *et al.* 2002; Rosser *et al.* 2002). The best functional improvements to date have been reported in a French cohort 2 years after transplantation on a range of neuropsychological tests and the UHDRS, corresponding with magnetic resonance imaging (MRI) signal indicative of surviving transplants, which stabilised for up to 6 years in some patients (Bachoud-Lévi *et al.* 2006; Bachoud-Lévi *et al.* 2000; Bachoud-Levi *et al.* 2000).

Post-mortem data from patients who died post-transplantation, though not as a result of the transplant, have shown graft integration and presence of relevant striatal markers within the graft (Capetian *et al.* 2009; Freeman *et al.* 2000). Over time the health of grafts has been reported to deteriorate, with minor infiltration of microglia (Cicchetti *et al.* 2009). However the analysis of the health of grafts here was carried out through comparisons between grafted cells and surrounding host tissue in transplants which were *in situ* for a long period of time (10 years) compared to recently transplanted cells (18 months - Cicchetti *et al.* (2009)). Since the surrounding host tissue in these cases could not be considered to be comparable, these findings may not be truly representative of the health of the transplants (Rosser and Bachoud-Lévi 2012). As no serious side effects or evidence of acceleration of the disease itself have been observed in transplant studies, ongoing work seeks to reduce variability in outcome through optimisation of transplant and immunosuppression protocols. See Dunnett and Rosser (2011) and Wijeyekoon and Barker (2011) for reviews of clinical studies carried out to date on hPF striatal transplantation in HD.

1.1.3 Alternative donor cells for transplantation

Clinical trials have demonstrated that cell replacement therapy can be effective in the treatment of neurodegenerative disease; however the use of hPF cells as a donor source for transplantation poses numerous problems. Laws vary in the collection of tissue from elective termination of pregnancy (ToP) due to the ethical issues surrounding the procedure. The UK requires local ethical approval for the collection of such tissue which, in Wales is carried out through the South Wales Initiative for Foetal Transplantation (SWIFT) programme, and this work is licensed by the human tissue authority (HTA). Additionally, the decision to donate foetal material for research is made by the maternal donor separately to that of the decision for ToP, with separation between medical and research teams and no option to donate tissue to a specific recipient (Polkinghorne 1989). A number of foetal donors are necessary for each patient; potentially up to 6 in HD, and 8-12 in PD, owing to the number of cells of a specific striatal or dopaminergic phenotype required (Barker and de Beaufort 2013; Rosser and Dunnett 2007). Since primary tissue cannot be stored prior to transplantation for more than a maximum of 8 days in hibernation medium (Hurelbrink *et al.* 2000), clinical transplantation would rely on the availability of sufficient donor tissue within this time. Due to the nature of the procedure, in which patients receive unique transplants from different donor foetuses, there can be problems in the variability and purity of cells which may affect the success of transplants (Barker and de Beaufort 2013; Björklund and Lindvall 2000; Kelly *et al.* 2011). Therefore as well as refining the transplantation method, current research aims to investigate alternative sources for potential transplant donor cells (Kim and de Vellis 2009).

Proof-of-principle for cell replacement therapies in neurodegenerative diseases including PD and HD has been achieved with allografts of PF tissue. Alternative donor tissue is required to consist of comparable cell types at relevant stages of development to achieve replacement of cells lost in disease-related degeneration. Additionally, a large stable population of cells is necessary, preferably not dependent on direct derivation from foetal donors, which can be quality controlled and standardised across transplants. A number of potential cell types exist including; xenogeneic porcine cells, foetal derived neural precursor cells (NPCs)

which may be expanded in culture, embryonic stem cells (ESC) and adult bone marrow mesenchymal stem cells (MSC), and most recently induced pluripotent stem cells (iPSCs) and induced neurons (iN) which may be generated from adult somatic cells such as skin fibroblasts. Each donor cell type is associated with various benefits and pitfalls, and all require extensive pre-clinical assessment prior to clinical trials of safety and efficacy in HD patients.

Xenogeneic donor cells

The use of xenogeneic tissue has been proposed as a potential source of cells for CNS transplantation, in particular porcine cells. Donor cells derived from porcine foetal tissues offer the potential for generation of PF striatal tissue of a specific donor age. This can be produced from inbred breeding stock under standardised, controlled conditions and with tissue collection and preparation undertaken to sterile good manufacturing practice (GMP) standards. Porcine tissue also offers comparable size and developmental time-course to the human brain. However issues with xenograft rejection must be considered. Humans and other Old World primates have natural antibodies to the α-galactosyl (α-Gal) epitope, which is expressed by many porcine cell types, including endothelial cells in foetal and adult brain (Sumitran *et al.* 1999). Therefore the transplantation of porcine tissue results in binding of these natural host antibodies to the graft and activation of the complement system. This causes hyperacute rejection of solid tissue grafts, and requires that grafts are derived from dissociated neural tissue containing fewer donor endothelial cells and permitting avoidance of hyperacute rejection (Brevig *et al.* 2008). However, even this preparation is not protected from rejection. A number of methods have been used to promote survival of porcine transplants in rodent hosts including; treatment with the immunosuppressant CsA (Pakzaban and Isacson 1994), anti-CD4 monoclonal antibodies (Wood *et al.* 1996), and masking donor major histocompatibility complex (MHC) by pre-treating porcine donor cells with an antibody to MHC-I (Pakzaban *et al.* 1995). Although immunological issues can be navigated through donor cell preparation and immunosuppression, additional concerns with the use of porcine tissues include the potential for transmission of zoonotic viruses, such as porcine endogenous retrovirus (PERV).

Despite these factors a number of preclinical studies have been conducted demonstrating that surviving intra-striatal transplants of PF porcine VM in rat hosts can form connections (Isacson *et al.* 1995) and produce functional recovery (Galpern *et al.* 1996). An initial clinical trial of unilateral transplantation of embryonic porcine VM in PD patients was carried out, with either CsA immunosuppression or masking of MHC on donor cells. This study showed no adverse effects or evidence of transmission of PERV, with no observed differences between immunosuppression treatments on the clinical outcomes measured and in graft survival assessed through *in vivo* imaging. Reported clinical benefit was moderate one year after transplantation (Schumacher *et al.* 2000). Post-mortem data have shown evidence of some graft rejection, even in a CsA treated patient (Deacon *et al.* 1997). Similar results have been reported from transplants into patients with HD (Fink *et al.* 2000). Although a promising source of tissue, issues of rejection have presented problems with the transplantation of porcine embryonic tissue, thus more optimum donor cell types continue to be investigated.

Currently, the cell types providing the most interest as potential donor cells suitable for transplantation in HD are stem cells. In the broad sense these are precursor cells which possess the ability for continuous renewal, although this can vary, and differentiation into many different lineages. Stem cell types currently under investigation for their potential use in transplantation include pluripotent cells such as ESC, derived from the inner cell mass of the blastocyst stage embryo, and more potency restricted stem cells derived from other sources including cord blood and bone marrow (MSCs).

Pluripotent Stem cells

A number of human ESC lines are available, including those of a GMP grade suitable for transplantation in the clinic. Protocols have been developed for the successful neural induction of human ESCs, allowing specification to the desired neuronal lineage (Chambers *et al.* 2009). Differentiation of ESCs directed to a dopaminergic phenotype has resulted in cells with some evidence of function following transplantation into animal models of disease (Rodríguez-Gómez *et al.* 2007). Protocols have also been developed for differentiation of ESCs into GABA-ergic striatal neurons, expressing MSN markers including DARPP-32 (Aubry *et al.*

2008; Carri *et al.* 2013; Ma *et al.* 2012; Parmar and Björklund 2012). Following transplantation into the QA lesioned mouse striatum of immunodeficient mouse hosts, these cells have shown expression of DARPP-32 and substance-P with some evidence of alleviation of motor deficits (Ma *et al.* 2012). An alternative protocol showed the same appropriate development of cells *in vivo* in immunosuppressed rat hosts following transplantation, however longer term follow up in immune compromised rats revealed problems with overgrowth of transplanted ESCs (Aubry *et al.* 2008). Moderate behavioural improvements have been reported with a different MSN differentiation protocol, however histological analysis at 6 and 9 weeks post-transplantation was again indicative of graft overgrowth (Carri *et al.* 2013).

This highlights an important issue with the use of ESC derived cells in transplantation, which also applies to the other cell types discussed in this section. Prior to transplantation of cells differentiated from a pluripotent cell type, adequate specification and reduction in pluripotency markers must be confirmed to reduce risks of tumorigenesis *in vivo*. These cells must then be adequately tested pre-clinically with long term assessments post-transplantation to determine the risk of overgrowth. Additionally, although ESCs can offer a source of cells with the potential for virtually unlimited proliferation and genetic modification (Steindler 2007), the cells still require derivation from embryonic tissue. This introduces ethical issues comparable to that of the use of PF donor tissue, in addition to legislation regarding the development and maintenance of stem cell lines from this donor tissue.

More recently, successful reprogramming of adult somatic cells to a pluripotent state has been achieved using a set of factors known to have a role in the maintenance of pluripotency in early embryos and ESCs; Oct3/4, Sox2 and Klf4, or increase proliferation in tumour cells; c-Myc (Takahashi and Yamanaka 2006). The resulting pluripotent cells may then be differentiated into required cell types for transplantation, as with ESCs. iPSCs have been successfully differentiated into neurons, and they offer the same advantages as ESCs, avoiding the need for foetal donors and also providing the additional potential for the generation of patient specific cell lines from adult fibroblasts (Takahashi and Yamanaka 2006). This raises the possibility of delivery of autologous transplants in patients, which may circumvent the need for immune suppression. However an investigation into the

rejection of mouse iPSC transplants in mouse hosts challenges this suggestion. Zhao *et al* sought to investigate the immunogenicity of iPSCs to determine whether such autologous transplants would be tolerated as assumed (Zhao *et al.* 2011). The authors showed that mESC derived from C57BL/6J (B6) mice could efficiently form teratomas following injection into B6 mouse hosts; whereas injections of miPSCs reprogrammed from B6 mouse embryonic fibroblasts (MEF) resulted in rejection accompanied by T cell infiltration. Following global gene analysis from teratomas formed by both cell types, several genes were reported to be overexpressed in miPSC derived teratomas which were shown to contribute to the immunogenicity of these cells (Zhao *et al.* 2011). Subsequent reports have disagreed with these findings and shown no immune responses to miPSC derived skin or bone marrow cells in B6 mouse hosts (Araki *et al.* 2013). Therefore, whether successful autologous transplantation without immunosuppression can be achieved has not yet been determined.

Concerns also derive from differences found in gene expression profiles of iPSC as compared to ESCs (Chin *et al.* 2009), and iPSC lines have been reported to develop epigenetic and chromosomal abnormalities with continued expansion *in vitro* (Pera 2011). Aside from these abnormalities; the identification that iPSCs retain some epigenetic memory for the original donor cell provides the potential to favour iPSC differentiation to a specific cell type, which may be used advantageously in developing differentiation protocols for cells for transplantation (Bar-Nur *et al.* 2011; Kim *et al.* 2010). Since cells are reprogrammed to a pluripotent state, and subsequently differentiated to the desired cell type, there are risks of persistent pluripotency from the reprogramming factors resulting in potential tumorigenesis following transplantation *in vivo*. Much work is required to characterise the differences between iPSC and ESC compared to PF tissue, and the various reprogramming methods available before they can be translated to the clinic, aside from the standard testing required to demonstrate safety and function pre-clinically.

Lineage restricted stem cells

The presence of multipotent cells in the developing brain gives the potential for the expansion of these cells in culture, providing a potential source of NPCs for

transplantation. NPCs may be derived from the foetal CNS and cultured with the addition of growth factors such as epithelial growth factor (EGF) and fibroblast growth factor (FGF-2), allowing proliferation of cells which are already committed to a neuronal lineage (Svendsen *et al.* 1998), or even a striatal lineage. Although this presents an attractive donor source for transplantation, it has been shown that following expansion in culture, changes in gene expression occur, and the longer cells are expanded in culture the less they are able to differentiate and survive after transplantation *in vivo* (Zietlow *et al.* 2005; Zietlow *et al.* 2012). Although transplanted NPCs have been reported to improve survival of endogenous neural cells through intrinsic neuroprotection properties via the release of growth factors and immunomodulatory molecules at the graft site (Pluchino *et al.* 2009), the development of the relevant cell type *in vivo* is still required for true circuit repair. Thus the main theoretical benefit of these cells (the ability for expansion in culture to provide a constant source of cells which are committed to the relevant neuronal lineage) does not appear to be a reality.

Following successful reprogramming of adult cells to a pluripotent state requiring subsequent neuronal differentiation; direct conversion of fibroblasts to neuronal cells has now been achieved (Vierbuchen *et al.* 2010). As these cells do not require differentiation from the reprogrammed pluripotent state, this avoids the issues associated with potential tumour formation. First demonstrated in mouse cells, three neural-lineage-specific transcription factors; *Ascl1*, *Brn2* and *Myt1l* (BAM), were expressed in mouse postnatal fibroblasts resulting in efficient conversion to functional iNs *in vitro* (Vierbuchen *et al.* 2010)). Successful generation of iN from human fibroblasts with the same factors and the addition of another factor, *NeuroD1*, was subsequently achieved (Pang *et al.* 2011). Additionally, combining the BAM factors with expression of *Lmx1a* and *FoxA2*, two genes involved in the natural generation of dopaminergic neurons, has resulted in the generation of directly induced dopaminergic neurons with potential for use in transplantation in models of PD or for disease modelling *in vitro* (Pfisterer *et al.* 2011).

Another lineage restricted stem cell type, MSCs, can be harvested from adult or foetal bone marrow, as well as a number of other tissues. These cells are characterised by plastic adherence, rapid proliferation and multipotency (Dominici *et*

al. 2006). As these cells can be derived from adult bone marrow, this could avoid some of the ethical and practical issues associated with the use of foetal tissue. Although reportedly only a low percentage of MSCs can differentiate into neurons following transplantation into the QA lesioned rat striatum (Lescaudron *et al.* 2003), the cells possess other properties of interest. Numerous studies have reported the immunomodulatory capabilities of MSCs, demonstrating suppression of T cell proliferation (Di Nicola *et al.* 2002; Zappia *et al.* 2005). On transplantation, MSCs can provide neurotrophic support and improve survival of co-transplanted adult neural stem cells (aNSCs) leading to behavioural improvements in a model of HD (Dunbar *et al.* 2006; Rossignol *et al.* 2014). These findings will be discussed in further detail in section 1.2.3.

The use of cells for transplantation from all stem cell types involves a number of common issues which must be addressed before translation to the clinic. First, differentiation into the appropriate neural phenotype must be confirmed, ensuring no pluripotent cells remain and no aberrant differentiation has occurred. Additionally, it must be determined whether producing and transplanting a pure population of cells for transplantation is adequate for survival of cells and repair in the host brain. For example, is it sufficient to produce a pure population of dopaminergic neurons to transplant in PD or will supporting cell types be required? PF tissue transplants have demonstrated success in both pre-clinical and clinical trials and this donor tissue will contain a heterogeneous population of cells including astrocytes and interneurons which will not be present in most stem cell derived neuronal populations. Thus it remains to be determined whether “support” cells may need to be present during differentiation of stem cells into the appropriate cell type, or added prior to transplantation to improve survival and functionality of transplants. In order to produce cells for clinical transplantation, protocols must be refined for adequate expansion to the number of cells required for patient transplants and all production will need to be carried out under controlled GMP approved conditions.

In order to assess the suitability of novel cell types for transplantation in human patients, donor cells must first be tested via xenotransplantation into animal models of disease to determine safety and efficacy, and to optimise cell preparation, implantation, immunological management and assessment procedures (Steindler

2007). The evaluation of the suitability of donor tissue requires histological examination of the integration of the cells to determine whether relevant connections have been formed within the host brain. Behavioural testing is also necessary to determine whether surviving cells are functional, and improvement is observed on a battery of relevant tests of cognitive and motor performance. These measures require the cells to be fully differentiated in the host brain. Due to differences in developmental time span between humans and rodents, human tissue will mature and differentiate more slowly *in vivo* (from 15-30 weeks - Brundin *et al.* (1986)), therefore any improvements will not be observed until a longer time after grafting. As xenotransplantation elicits a strong immune response from the host, grafts are rejected fairly quickly in animals in the absence of immunosuppression. Xenografts in the rodent brain are usually rejected by around 20-30 days (Barker *et al.* 2000; Mason *et al.* 1986; Sloan *et al.* 1991), dependent on the type and location of the graft. Treatment is therefore required to prevent rejection by the host immune system. Currently, CsA is the most common method of immunosuppression used, however this requires daily injections and causes severe side effects in the animals which usually lead to termination of the experiment at around 12-16 weeks post transplantation; insufficient time for full differentiation of transplanted human cells. This creates a problem for the adequate pre-clinical assessment of potential donor cell types for neural transplantation in patients, as no current immunosuppression method permits full safety and functional assessment of the cells.

1.2 The immunological response to neural xenografts

The immune system in all species serves a common function; to protect the organism against potential threats, whether this may be from pathogens such as bacteria and viruses, or foreign cells as in the case of transplantation. In order to identify and eliminate potential threats, the immune system must possess the specific ability to determine 'self' antigens from 'non-self' antigens. This phenomenon forms the basis of all immune responses; in the identification of foreign antigens, their removal, and the development of immunity. Following transplantation, the donor cells are the source of foreign antigen presented to the immune system. In the normal, healthy immune system, these are targeted by the host as 'non-self' antigens for elimination. Examples of failures in this 'self'-'non-self' discrimination are found in autoimmune and immunodeficiency disorders. In autoimmune diseases such as multiple sclerosis (MS) or diabetes, the patients' own antigens are not recognised as 'self' and are therefore targeted by their immune system. Conversely the immune system in immune-deficient individuals has a compromised or absent ability to stave off infection.

In the case of transplants to the CNS; historically this was thought to be a site of some immunological privilege. It is now clear, however, that the rejection of neural transplants in immunologically competent hosts does occur, albeit to varying extents dependent on the type of transplant. The immune system of the CNS will therefore be discussed in further detail in this section, along with the mechanisms of rejection of cells transplanted into the brain. Although the issues of the immune response to allografts in clinical CNS transplants must be considered and elements of this section are relevant here, the focus of this discussion is on the rejection of pre-clinical transplants in animal models.

1.2.1 The immune system in the CNS

The brain has been traditionally considered to be an "immunologically privileged site" (Barker and Billingham 1978; Medawar 1948) due to the increased survival of cells transplanted to the CNS compared to the periphery. This was attributed to a number of properties of the CNS thought to provide relative protection from the immune system in comparison to other tissues. The presence of an

endothelial blood brain barrier (BBB) sealed by tight junctions restricts the diffusion of larger molecules from the blood into the brain and therefore prevents infiltration from circulating immune cells (Goldstein and Betz 1983; Mark Richardson *et al.* 2005; Zappia *et al.* 2005). There is also a lack of professional antigen-presenting cells (APCs) (Laguna Goya *et al.* 2011) in the healthy CNS. Professional APCs; including dendritic cells (DCs), B cells and macrophages, are required to process and present antigen to T cells via MHC molecules in order to initiate an immune response (Wekerle *et al.* 1987). The lymphatic drainage from the CNS to the immune system itself is poor, also preventing the presentation of foreign antigens in the periphery (Barker and Widner 2004), although this has been disputed, suggesting there is an unconventional form of lymphatic drainage which can transport activated immune cells to the periphery (Hatterer *et al.* 2006). Additionally, neural cells exhibit very low expression of Class I (Joó 1993; Wekerle *et al.* 1987) and no expression of Class II MHC antigens (Einstein *et al.* 2007; Goldstein and Betz 1983; Martino and Pluchino 2006), thus providing some protection from recognition by host T cells (Barker and Billingham 1978). However, recently more has been discovered about the immune surveillance of the brain. The CNS is now considered to be a region of 'relative' immunological privilege (Galea *et al.* 2007); although transplanted cells are provided some protection, the activation of an immune response can still initiate graft rejection. Therefore, without immune suppression, long term survival of neural transplants is compromised.

The mechanisms which offer such immunological privilege to neural cells transplanted into the brain only apply in the context of the normal, healthy CNS. Since the penetration of the brain and implantation of cells initiates an inflammatory response and damages the BBB itself, the immunological surveillance of the CNS will no longer be restricted and circulating immune cells may be able to enter the brain (Brundin *et al.* 1989; Finsen *et al.* 1991). In addition to BBB damage through surgery; it is now known that under inflammatory conditions the BBB becomes permeable to activated lymphocytes, allowing infiltration of T cells into the brain (Hickey 2001; Hickey *et al.* 1991; Kebir *et al.* 2007; Laguna Goya *et al.* 2011; Schmidt-Kastner *et al.* 1993). The loss of protection from the BBB is sufficient to promote recognition of donor cells by host immune cells, as demonstrated by Pollack *et al*, who showed that established grafts implanted in the neonatal period are

rejected following disruption of the BBB in adulthood as a result of an influx of immune cells (Pollack and Lund 1990). Transplants to the brain are therefore susceptible to rejection without immune suppression, and although there is some protection offered to the brain in comparison to other tissues, this is not absolute (Gorelik *et al.* 2012).

Local trauma to the grafted area also promotes the release of pro-inflammatory cytokines increasing the permeability of the BBB and providing another mechanism for the ingress of immune cells into the brain. Studies have shown variability in the length of time for recovery of the BBB following transplants (Sanberg *et al.* 1988; Wakai *et al.* 1986). This may be due to variations in the inflammatory conditions around the graft; although the initial damage to the BBB from surgery has been repaired, increased permeability may continue. Demonstrating this, transplants have been shown to increase permeability of the BBB for a longer period of time than a lesion or vehicle injection (Sanberg *et al.* 1988). In the development of an inflammatory response; cytokines including tumour necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-6 (IL-6) are produced by macrophages, microglial cells, astrocytes and cortical epithelial cells (CECs) in the CNS (de Vries *et al.* 1997). The release of such cytokines is thought to increase the permeability of the BBB and this effect has been demonstrated *in vitro*, with the administration of TNF-α, IL-1 and IL-6 to endothelial cell models of the BBB increasing transport across the barrier (de Vries *et al.* 1996; Deli *et al.* 1995). This finding has been confirmed *in vivo* a number of times; see de Vries *et al.* (1997) for a review. Additional cytokines including IL-17 and IL-22 have also been shown to affect the permeability of the BBB through disruption of tight junctions both *in vitro* and *in vivo* following their release by T helper (TH)17 lymphocytes, a subset of TH cells (Kebir *et al.* 2007). An opposing effect has been found for transforming growth factor-β (TGF-β), which is present in cerebrospinal fluid (CSF) and also inhibits MHC-II expression on APCs (See Fabry et al 1995). TGF-β has been shown to reduce leukocyte migration across CNS endothelial cells *in vitro* and through the BBB *in vivo* (Fabry *et al.* 1995) and its presence is known to suppress proliferation of T cells which may have passed through into the brain.

1.2.2 Neural transplant rejection

The immune response to neural grafts involves a circuit with both afferent and efferent arms. The afferent arm of the immune response includes the recognition of antigens via antigen presentation, causing activation of lymphocytes and the generation of effector cells. Foreign antigenic material is presented to T cells by APCs (Sloan *et al.* 1991). In the resting state no professional APCs are present in the brain, however during inflammation resident microglia are capable of maturing into macrophages and dendritic cells (DCs); professional APCs which express MHC antigens and accumulate within the brain parenchyma (Santambrogio *et al.* 2001). Although donor cells both from the developing brain and host cells in the adult brain show low or no expression of MHC Class I or II, upregulation of both can occur following exposure to inflammatory signals in response to transplantation (Litchfield *et al.* 1997; Pakzaban and Isacson 1994). The up-regulation of Class I and II MHC molecules in neural tissue is induced by pro-inflammatory cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α). Class II expression allows this afferent arm of the immune response to take place through recognition by $CD4^+$ TH cells (Pakzaban and Isacson 1994). The presentation and processing of antigens requires the formation of MHC/antigen peptide complexes bound to the T cell receptor (TCR), known as signal 1 (see **Figure 1.1** for a diagrammatic representation of this complex).

Additionally, the formation of complexes by costimulatory molecules on APCs and their receptors on the surface of T cells (e.g. B7-CTLA4 and CD40-CD40L) is required for activation of the T cell (Barker and Widner 2004). These complexes are also shown in **Figure 1.1**. This is known as signal 2, with activation of signal 1 alone insufficient to trigger a clonal expansion, survival and differentiation of activated T cells (Lafferty and Cunningham 1975). The activation of T cells by TCR stimulation in the absence of this second signal can result in anergy or apoptosis of the responding T cell (June *et al.* 1990; Schwartz 1990). A number of costimulatory molecules exist which may support or inhibit T cell activation. **Table 1.1** is not an exhaustive list, but includes well characterised costimulatory pairs implicated in transplant rejection (Kinnear *et al.* 2013). Four distinct groups of costimulatory families can be classified based on their structure; Ig superfamily members such as

CD28; TNFR family members such as CD40; cell adhesion molecules or integrins; and T cell Ig domain and mucin domain (TIM molecules (Kinnear *et al.* 2013). The best characterised are the Ig and TNFR families, some of which are represented in **Table 1.1** and are discussed here.

The first costimulatory pathway to be defined, and the most well characterised,

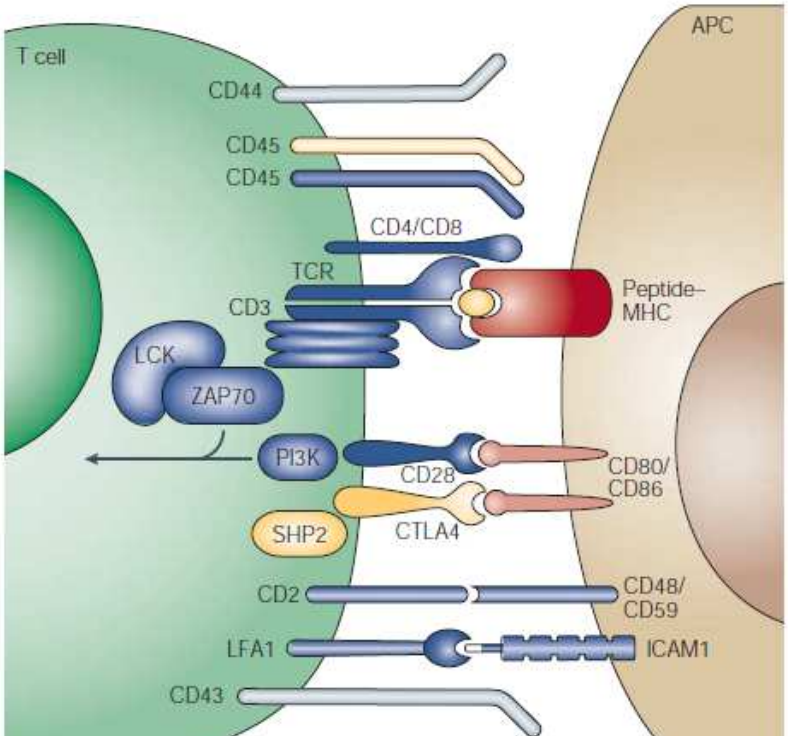

Figure 1.1 Key ligand pairs and signalling molecules involved in T-cell recognition. Peptide-MHC molecule = red. Activating/co-stimulatory molecules = blue. Inhibitory molecules = yellow. Reproduced from Huppa and Davis (2003)

is the B7/CD28/CTLA-4 pathway. CD28 is constitutively expressed on around 80% of human naïve T cells (around 50% of $CD8^+$ and all $CD4^+$), and almost 100% of murine T cells. Its expression is also increased following T cell activation (June *et al.* 1990; Lenschow *et al.* 1996). The ligands for CD28; the B7 molecules CD80 (B7-1) and CD86 (B7-2) are expressed on APCs. CD80 expression is induced following activation on APCs and activated T cells, whereas although CD86 is constitutively expressed by APCs, its expression is rapidly upregulated following activation (Greenwald *et al.* 2005). In the presence of TCR stimulation (signal 1), costimulation via CD28 and the B7 ligands lowers the threshold for T cell activation and increases the expression of IL-2; which in turn promotes growth and proliferation of T cells to

become effector T cells (Lenschow *et al.* 1996; Wood and Goto 2012). Costimulation via CD28 also leads to the up-regulation of CTLA-4, and other costimulatory molecules including CD154 (CD40L) (Wood and Goto 2012). The function of these molecules is discussed below.

An additional receptor for the B7 molecules, CTLA-4, is upregulated on T cells following activation and has a higher binding affinity for CD80/CD86, up to 10 to 20 times greater than CD28 with a preference for CD80 (Wood and Goto 2012). CTLA-4 is also constitutively expressed by FoxP3 regulatory T cells (Tregs), with a role in Treg cell mediated suppression demonstrated by failure to activate Tregs and a lack of this suppression when CTLA-4 is blocked (Sakaguchi 2004). Following upregulation on activated T cells, CTLA-4 competes with CD28 for ligation with the B7 molecules, which limits CD28/B7 interactions resulting in decreased IL-2 secretion and attenuation of the T cell response (Walunas *et al.* 1994). Loss of CTLA-4 has therefore been shown to be lethal in mice, resulting from massive proliferation of lymphocytes (Tivol *et al.* 1995). The balance between costimulation from CD28 and CTLA-4 is therefore required for T cell activation and prevention of the continuation of this response (Alegre and Najafian 2006). This interaction has provided a method attempting to induce tolerance to transplants, via blocking costimulatory molecules and disrupting these pathways, which is discussed further in section 1.3.2.

Costimulatory molecule	Receptor expression	Ligand	Ligand expression	Function
CD28	Naïve T cells	B7-1 (CD80),	APC (induced upon activation)	Stimulatory
		B7-2 (CD86)	APC (constitutively)	
CTLA-4 (CD152)	Naïve T cells, Tregs	B7-1 (CD80)/B7-2 (CD86)		Inhibitory
ICOS (CD278)	Activated T cells	ICOSL	B cells, monocytes	Stimulatory
PD-1 (CD279)	Activated T cells, B cells and myeloid cells	PD-L1	Resting T cells, B cells, DC, macrophage, endothelial cells	Inhibitory
	DC macrophages	PD-L2		
OX40 (CD134)	Activated T cells	OX40L	B cells, macrophages, DC, vascular endothelial cells, mast cells, activated NK cells	Stimulatory
41BB (CD137)	$CD4^+$ and $CD8^+$ NK cells	41BBL	Mature DC, activated B cells and macrophages	Stimulatory
CD40	B cells, DC, macrophages	CD40L (CD154)	T cells, B cells, activated platelets, DC, esosinophils	Stimulatory
CD27	Activated T and B cells, NK cells	CD70	Activated T and B cells, NK cells, DC	Stimulatory

Table 1.1 Costimulatory pairs involved in the immune response to transplants. Adapted from (Kinnear *et al.* 2013; Li *et al.* 2009). Well characterised costimulatory molecules are listed with their ligands and the cells on which each are expressed, showing whether costimulation results in inhibition or stimulation of an immune response.

CD40 and its ligand CD154 (CD40L) belong to the TNF(R) superfamily and their interaction results in activation of an immune response via activation of DCs (Yang and Wilson 1996). Although expressed constitutively at low levels on APCs, CD40 is significantly upregulated following cellular activation, including upregulation by CD28 expression on T cells, and its ligand CD154 (CD40L) is only expressed on activated immune cells (listed in **Table 1.1**). The subsequent activation of DCs results in further upregulation of costimulatory molecules and production of inflammatory cytokines promoting antigen presentation to T cells (Yang and Wilson 1996). CD40 is also an important costimulatory molecule for B cells, inducing the proliferation and production of antibodies (Alegre and Najafian 2006).

The immunological synapse formed through the binding of TCRs and MHC peptides on surfaces of APCs, and the ligation of costimulatory molecules lead to a third signal which produces a number of intracellular processes. These result in altered gene expression leading to upregulation of IL-2 and promotion of cell cycle progression and clonal expansion and differentiation of activated T cells (Wood and Goto 2012). Within this process, TCR-MHC-peptide engagement leads to recruitment and phosphorylation of several signalling molecules which ultimately leads to upregulation of IL-2. This generation of large amounts of IL-2 acts in an autocrine and paracrine way to create “signal 3”, promoting cell cycle progression and initiating clonal expansion and differentiation of activated T cells (Wood and Goto 2012). **Figure 1.2** shows the pathways involved in signal 3, leading to T cell activation and expansion. This pathway will be relevant to the mechanism of action of immunosuppressant drugs discussed in section 1.3.1.

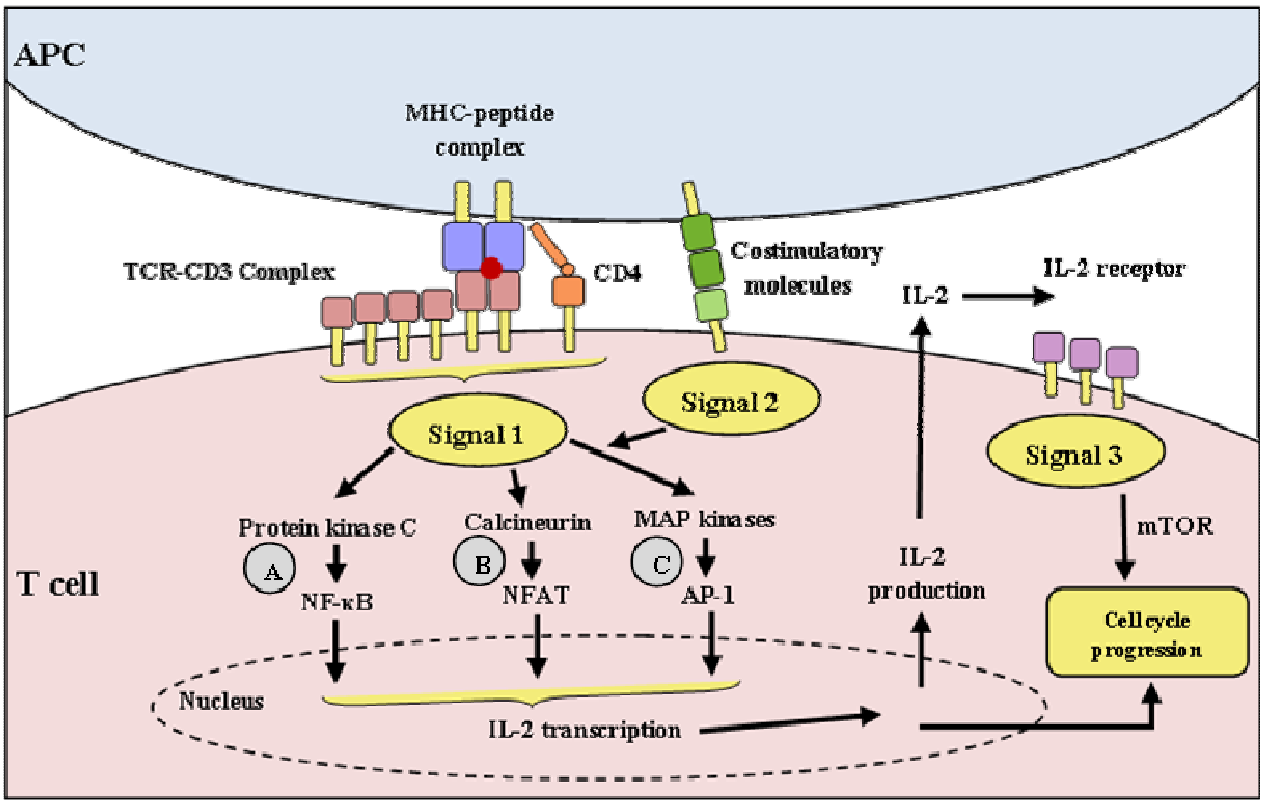

Figure 1.2 Diagrammatic representation showing a simplified outline of signalling molecules involved in "signal 3", adapted from Wood and Goto (2012). Phosphorylation events result in activation of protein kinase pathways and the generation of secondary messengers IP_3 and diaglycerol (DAG). Generation of DAG activates the transcription factor nuclear factor kappa B (NF-κB) (A). IP_3 leads to the release of stored calcium and activation of calcineurin which dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT), allowing translocation to the nucleus (B). Mitogen-activated protein (MAP) kinase cascades generate the third transcription factor; AP-1 (C). Activation of these transcription factors leads to upregulation of IL-2 which acts in an auto- and paracrine way to provide signal 3.

Following transplantation, antigen presentation may occur through a direct or indirect pathway. Direct recognition requires host T cells to identify MHC molecules expressed on donor cells, whereas indirect recognition occurs following the breakdown and presentation of donor peptides by MHC molecules on the host's own APCs (Gould and Auchincloss 1999). Both direct and indirect presentation of antigens may have a role in xenograft rejection (Pakzaban and Isacson 1994). However it has been suggested that the indirect pathway is more important, since species differences between antigens of donor origin and the T cell receptors of the host may prevent recognition of directly presented donor MHC peptide complexes (Gill and Wolf 1995). Indeed, the relative contribution of indirect recognition is known to increase with greater species disparity (Auchincloss and Sachs 1998; Gill and Wolf 1995). Additionally, over time the number of donor cells within the graft with the ability to present antigen diminishes, reducing the role of the direct recognition pathway. However as long as the graft is present, the indirect pathway may present antigen and is therefore the dominant pathway in the long term (Wood and Goto 2012).

Activation of T cells through antigen presentation and co-stimulatory molecule interaction results in proliferation of T cells specific for these antigens, and the generation of effector-cell populations which migrate to the graft site and cause graft rejection (Sloan *et al.* 1991). See **Figure 1.1** for a diagrammatic representation of the T cell synapse and interaction with APCs (Huppa and Davis 2003). Lymphatic drainage from the brain through the movement of CSF allows the delivery of antigenic material to the peripheral lymphoid organs where immune competent cells can initiate an immune response (Cserr *et al.* 1992). Resulting responses from pro-inflammatory cytokines such as interleukin-1 (IL-1) and IFN-γ promote proliferation of T lymphocytes (Hickey 2001). The release of TNF-α also allows an increase in the permeability of the BBB, allowing activated T cells to traverse into the brain (Barker and Widner 2004). This encompasses the efferent arm of the immune response, induced by expression of MHC-I allowing targeting by CD-8^+ effector T cells (Pakzaban and Isacson 1994). This requires the passage of activated lymphocytes into the brain due to the increased permeability of the BBB by

proinflammatory cytokines and specifically vascular cell adhesion molecule-1 (VCAM-1) (Merrill and Murphy 1997).

In the case of neural xenografts, infiltration of host immune cells has been shown to primarily consist of macrophages and T cells, with only a low number of B cells (Finsen *et al.* 1991; Pedersen *et al.* 1997). During rejection, host DCs infiltrate the transplant along with these immune cells (Lawrence *et al.* 1990). The presence of $CD4^+$ T cells is required for the rejection of xenografts, as shown by the use of CD4 monoclonal antibodies for long term prevention of neural xenograft rejection (Wood *et al.* 1996). In addition to the cellular, T cell mediated aspects of neural xenograft rejection; it is possible that the humoral arm of the immune system is also activated during the host response. If pre-existing antibodies to donor tissue are present within the host; a rejection response can follow transplantation immediately, known as hyperacute rejection. This has been discussed in section 1.1.3 in the use of porcine tissue for transplantation, to which humans possess preformed antibodies which react to α-Gal. The development of antibodies specific to donor MHC antigens has been observed in some animals following the striatal transplantation of mouse VM (Brundin *et al.* 1989), however the disparity between these species is low. Confirmation of the involvement of a humoral response in more distant species has been shown in the delay in rejection of porcine neural tissue transplanted into immunoglobulin knockout (IgKO) mice as compared to wild type controls. The authors concluded that Ig have a role in the initiation of xenograft rejection. Grafts in IgKO mice were found to survive up to 4 weeks, at which stage infiltration of both $CD4^+$ and $CD8^+$ T cells resulted in graft rejection (Larsson *et al.* 1999). The interaction between the humoral and cellular immune responses in xenografts has been examined by Barker et al (Barker *et al.* 2000), who found that the inhibition of complement also reduces the cellular response. The involvement of complement and humoral responses provides an explanation for the inconsistent prevention of xenograft rejection with CsA treatment daily (Pakzaban and Isacson 1994), however the relationship between these humoral and cellular responses remains unclear.

1.2.3 The effect of donor cell type and transplant location

Correlations have been demonstrated between the level of MHC expression and graft rejection, with high levels of MHC-I expression relating to rejection of

transplants, and low levels relating to transplant survival (Mason *et al.* 1986). However the level of MHC expression on donor tissue is not the only factor relating to transplant rejection. Additionally, the lack of MHC expression on allogeneic NPCs has been shown to activate murine natural killer (NK) cells, which subsequently target transplanted cells for rejection (Phillips *et al.* 2013). NPCs from foetal brain have been reported to survive following transplantation to non-immune-privileged sites (under the kidney capsule) for up to 28 days post-transplantation, as compared to transplants of mature neurons which were rapidly rejected (Hori *et al.* 2003). It has therefore been assumed that some characteristics of these cells protect them from recognition by the host immune system following transplantation. It was shown that hosts were not tolerant to these transplants, since they could be rejected with sensitisation to donor tissue pre- or post-transplantation, therefore NPCs were presumed to survive due to a lack of sensitisation to donor tissue (Hori *et al.* 2003).

Human foetal neural tissues have been shown to express low or no MHC, however following time in culture both MHC class I and II are up-regulated on NPCs (Odeberg *et al.* 2005). Despite this, NPCs were not reported to trigger an immune response when investigated *in vitro* by lymphocyte proliferation co-cultures. This is thought to be due to the fact that, although MHC expression was increased in NPCs after long-term expansion *in vitro*, expression of co-stimulatory molecules was not present (Odeberg *et al.* 2005). Transplantation studies have also suggested NPCs to be less immunogenic than primary tissue in porcine to rat xenografts, although these findings require assessment at further time-points post-transplantation (Armstrong *et al.* 2002). Up-regulation of MHC expression following time in culture has also been shown in rat NPCs (McLaren *et al.* 2001). A subsequent study has shown that although neural tissue from human foetal cortex and VM showed low MHC class I expression, following proliferation in culture the expression increased rapidly. However further investigation showed that this increased expression was possibly related to a change in cell type within the culture towards a glial phenotype, and that MHC class I was only found to be expressed on NPCs, glial progenitors, mature astrocytes and oligodendrocytes, but not on neurons (Laguna Goya *et al.* 2011). This study, however, showed proliferative T cell responses in co-cultures with NPCs after a delay and attributed this delay to the contradictory findings with

previous studies, suggesting that this is why NPCs can still elicit rejection *in vivo* (Laguna Goya *et al.* 2011; McLaren *et al.* 2001; Odeberg *et al.* 2005).

As with NPCs, an investigation into the immunogenicity of ESCs reported low levels of MHC Class I and II in undifferentiated cells (Drukker *et al.* 2002). Subsequent differentiation of ESCs elevated expression of MHC-I but not II, however the addition of IFN-γ was shown to upregulate both. Therefore, it is assumed that this upregulation of MHC would be possible *in vivo* following transplantation (Drukker *et al.* 2002). Following this study, analysis of the mouse immune response to human ESC kidney capsule transplants was characterised to determine the effect these findings had *in vivo*, with transplants into immunodeficient hosts as a control (Drukker *et al.* 2006). A number of different mouse strains were transplanted, with complete rejection of hESC transplants after 1 month observed in immunocompetent mice. Transplants in mouse hosts which were deficient of T cells were not rejected, whereas no difference was found in mice deficient of B or NK cells as compared to immunocompetent hosts; indicating a role for T cells in xeno- rejection of hESCs. Transplants into humanised mice were not rejected, demonstrating allograft survival of ESC-derived transplants (Drukker *et al.* 2006).

As discussed in section 1.1.3; iPSCs have been reported to show differences in gene expression as compared to ESCs (Chin *et al.* 2009). Initially, iPSCs were thought to provide an optimum solution to the need for new donor cell types, in particular because cell lines could be developed to provide autologous transplants, or at least with banks of appropriate human leukocyte antigen (HLA)-typed cell lines. However more recent findings have shown unexpected variable immunogenicity in cell lines. Zhao et al found that autologous iPSC transplants were not tolerated in mouse hosts as predicted, and were infiltrated with T cells and rejected (Zhao *et al.* 2011). These findings have subsequently been disputed by Araki et al, who found no rejection of their autologous iPSC derived transplants (Araki *et al.* 2013), however it is clear that there are genetic and chromosomal abnormalities in iPSC lines which must be considered when developing these cells for CNS transplantation. It has been proposed that differences between reprogramming methods determine this immunogenicity, with iPSCs generated using viral vectors proposed to be more

vulnerable to rejection than those derived via episomal approaches (Boyd *et al.* 2012).

Some differences in immunological responses following transplantation of stem cells may be used advantageously for cell replacement therapies. In particular; as described in section 1.1.3, MSCs have been shown to possess a number of immunomodulatory functions, as well as the other benefits to the graft environment following transplantation. Suppression of T cell proliferation by MSCs has been demonstrated *in vitro* and shown to be dependent on both cell to cell contact and the release of soluble factors (Di Nicola *et al.* 2002; Zappia *et al.* 2005). As the release of an array of cytokines and trophic factors has been found from MSCs (Haynesworth *et al.* 1996), these cells have been hypothesised to have the ability to suppress inflammatory responses following transplantation. Following injection, they have been reported to block the inflammatory and T cell response, preventing maturation of monocytes into DCs and preventing antigen presentation to T cells (Chiesa *et al.* 2011; Pluchino and Cossetti 2013). Although not necessarily providing an optimum cell source for neuronal differentiation; co-transplantation of MSCs with neural cell types such as adult neural stem cells has shown protection by MSCs can promote survival of transplanted neural cells in a rat model of HD (Rossignol *et al.* 2014)

Aside from the immunogenicity of donor cells, the extent of the immune response may vary dependent on a number of factors, including the disparity between donor and host, the preparation and delivery of donor cells, and the location of the graft (Barker and Widner 2004; Wood and Goto 2012). The transplantation of solid tissue grafts generates a more severe and rapid response due to the intact vessels within the tissue demanding the formation of connections with the host vasculature and the activation of complement-mediated hyperacute rejection (Auchincloss and Sachs 1998; Broadwell *et al.* 1992). Additionally the method of delivery may have a variable effect on the inflammatory response due to damage to the BBB (Brandis *et al.* 1997). The phylogenetic distance between donor and host also dictates the contribution of humoral and cellular host immune responses to xenografts; this may be due to the degree of difference between donor and host MHC (Mason *et al.* 1986; Pakzaban and Isacson 1994). As mentioned previously there

may be some innate antibodies which can induce rejection of xenogeneic donor tissue following transplantation, as in the case of porcine to human xenotransplants discussed in section 1.1.3.

Reports have also suggested that younger donor tissue has an improved chance of survival (Brevig *et al.* 2008), due to a lower population of microglial precursor cells which may express MHC and act as APCs (Dalmau *et al.* 1997). The location of delivery of transplants can have an effect on the immune response, for example those transplants delivered closer to the ventricles are reported to be more at risk to rejection due to drainage of the subarachnoid space to cervical lymph nodes (Kida *et al.* 1993), the lack of BBB and the presence of MHC-II+ macrophages in the choroid plexus and the subarachnoid space (Vass and Lassmann 1990). In this context it is also relevant to consider the status of the host brain prior to transplantation, for example following previous damage to the system such as a lesion, or as a result of disease processes. Previous findings have suggested that transplants delivered just a few days after a QA lesion have improved survival as compared to those delivered after a week or more, due to the microglial environment in the graft site (Johann *et al.* 2007).

1.3 Immune suppression

A number of methods may be used to evade the host immune response to neural xenografts, including treatment of the host with drugs or antibodies, transplantation into neonates or immune compromised hosts. Additionally modifications to the donor cells themselves have attempted to mask them from the host immune system, and attempts have been made to induce tolerance to donor tissue. However as yet no method has been successfully optimised which allows full functional testing of potential human donor cells for a range of diseases. This is a problem not only for cell transplantation strategies in neurodegenerative diseases, but also for a number of others including amyotrophic lateral sclerosis (ALS), stroke, MS and spinal cord injury. The following discussion is not exhaustive, but covers a range of commonly used methods to promote the survival of xenografts in rodent hosts.

1.3.1 Immunosuppressant drugs

Currently, the most commonly used immunosuppression regime for the xenotransplantation of human cells into rodent models is the use of immunosuppressant drugs. A number of drugs are available which are used clinically for preventing rejection of organ transplants. Some immunosuppressant drugs which are used in pre-clinical transplant experiments in animal models of disease for testing transplants of xenogeneic donor tissue are listed in **Table 1.2**, including their mechanism of action. Treatment with one or a combination of these drugs can promote survival of cells transplanted to the CNS, although much variability has been reported as to the success of these treatments in different hosts and with the transplantation of different cell types. One of the most regularly used drugs currently is CsA, an immunophilin ligand which binds cyclophillin, blocking the phosphatase activity of calcineurin which is essential in T cell activation, thus preventing the initiation of an immune response (Ho *et al.* 1996). The mechanism of action of CsA, and other drugs discussed here are shown in **Figure 1.3** in the context of the immunological synapse. CsA and other immunosuppressant drugs are administered individually, or often in combination therapies to attempt to improve transplant survival (Sevc *et al.* 2013).

Immunosuppressive drug	**Mechanism of immunosuppressive action**	**Cell target (molecule)**
Azathioprine	Dampens proliferation of rapidly dividing cells	Many purine synthesis pathways (competitive inhibitor)
Mycophenolate mofetil	Dampens proliferation of rapidly dividing cells	Guanosine base synthesis (inosine monophosphate dehydrogenase)
Corticosteroids (e.g. prednisone)	Suppresses costimulatory signals (IL-1 and IL-6 platelet activating factor, prostaglandins, leukotrienes and TNF), oxygen burst and chemotactic and cytotoxic activities	APCs (IκB kinase)
Cyclosporine (CsA), tacrolimus (FK506 – TAC)	Blocks promoters of gene transcription, such as NFAT	Lymphoid cells (calcineurin)
Sirolimus (rapamycin)	Prevents dissociation of lκB and cytokine-driven G_1 accumulation	Costimulatory pathway for the production of cytokines and signal transduction after cytokine signalling (mammalian target of rapamycin, MTOR)

Table 1.2 Commonly used immunosuppressant drugs for preventing rejection of neural transplants including their mechanism of action and cell targets. Adapted from (Kahan 2003)

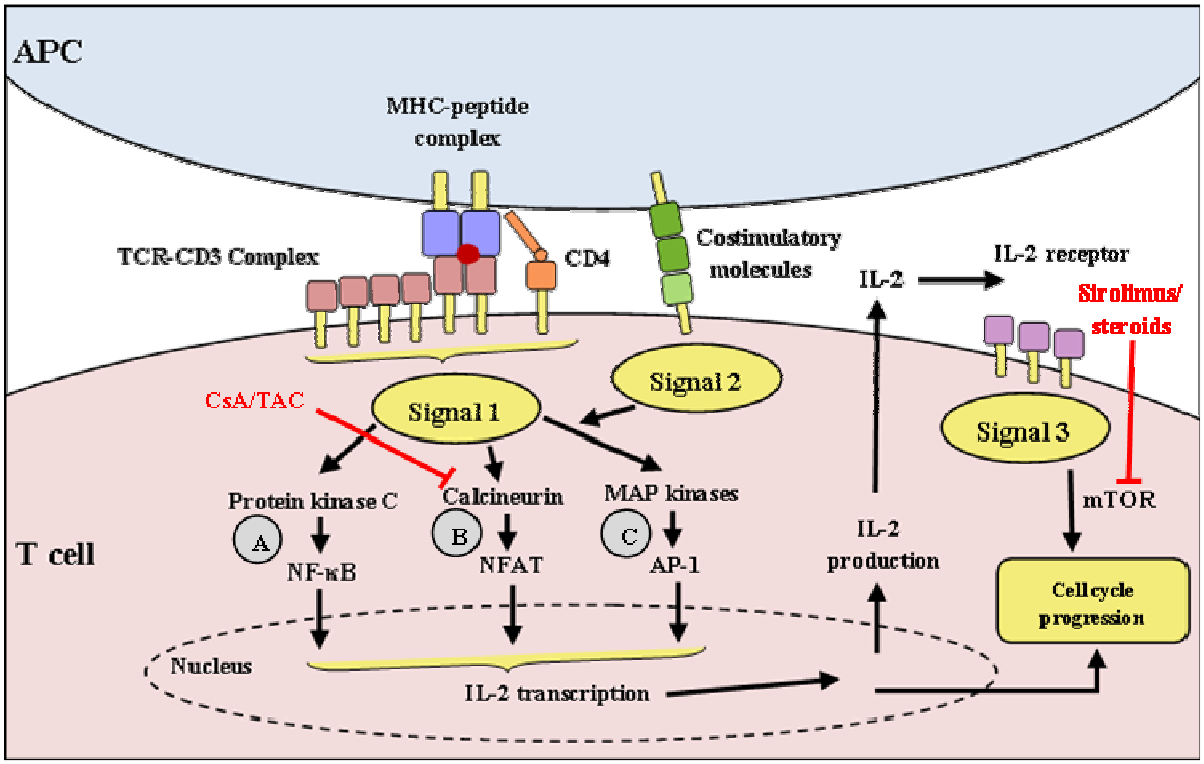

Figure 1.3 Mechanism of action of common immunosuppressant drugs, indicated in red. Cyclosporine (CsA) blocks phosphatase activity of calcineurin via binding to cyclophillin. Tacrolimus (TAC) binds FK binding protein 12 (FKBP-12), also blocking phosphatase activity of calcineurin. These both disrupt translocation of NF-κB to the nucleus and subsequent transcription of IL-2, thus blocking the initiation of T cell proliferation. Sirolimus binds FKBP-12, but this complex binds mammalian target of rapamycin (mTOR), inhibiting response to IL-2 and preventing cell cycle progression. Diagram adapted from (Wood and Goto 2012)

Although frequently used for immunosuppression in rat hosts, a number of studies have reported variability in graft survival in hosts treated daily with CsA (Jablonska *et al.* 2013; Larsson *et al.* 2001b). In our hands, CsA provides survival of human neural xenografts up to 75-80% in rat hosts (Kelly *et al.* 2009b) up to an absolute maximum of 20 weeks after transplantation due to toxic side effects of the drug. Although effective, this method requires daily injections of the drug which can be stressful to both the animal and the experimenter, and the treatment is associated with severe adverse side effects with long term use including renal toxicity (Al Nimer *et al.* 2004; Bertani *et al.* 1987). Survival can be potentially assessed up to 20 weeks, however, beyond 16 weeks the health of the animals starts to deteriorate, meaning functional testing is not possible.

An alternative immunophilin ligand which also inhibits the phosphatase activity of calcineurin is tacrolimus (FK506, TAC). Tacrolimus binds to FK-binding protein, specifically FKBP-12. The formation of this complex, like CsA, inhibits calcineurin (Snyder *et al.* 1998), however TAC has been reported to be 10-100 times more potent in its inhibition of calcineurin (Kino *et al.* 1987). Finally, sirolimus (rapamycin) also binds to FKBP-12, but does not to block the activity of calcineurin, this complex inhibits mTOR by direct binding of mTOR1, inhibiting translation and response to IL-2, and preventing T cells from progressing from G1 to S phase of the cell cycle (Alemdar *et al.* 2007). Both of these drugs have been reported to have neuroprotective side effects, making them promising treatments for immunosuppression in CNS transplants (Alemdar *et al.* 2007). Treatments with both TAC and CsA have been reported to increase the survival of dopaminergic cells *in vitro*, and following transplantation *in vivo* (Castilho *et al.* 2000). Increases in neurite length and branching have also been observed following treatment with immunophilin ligands; with those that inhibit the phosphatase activity of calcineurin (CsA, FK506), inducing elongation of dopaminergic neurites, and those which did not (sirolimus) increasing branching (Costantini and Isacson 2000). However, as with CsA, these drugs can also induce severe side effects making their effective use in pre-clinical transplant studies a challenge. Systemic administration of TAC for 14 days in mice to prevent the rejection of xenogeneic rat VM transplants resulted in good survival of transplants at 14 days post transplantation, and in surviving hosts at 28 days post-transplantation, however death of host mice was reported in a large amount of host animals following withdrawal of immunosuppression due to severe toxic effects (Sakai *et al.* 1991).

Side effects of these drugs include nephrotoxicity and hepatotoxicity (Finn 1999; Sevc *et al.* 2013), and due to the short drug half-life at least once daily administration is necessary. Alternative preparations of drugs have been investigated to try and alleviate some of the side effects without affecting graft survival, and to improve the administration regimen of the drug. Alemdar *et al* tested the efficacy of liposomal preparations of TAC and sirolimus individually and combined, when transplanted directly with xenogeneic tissue (Alemdar *et al.* 2007). Rat hosts with 6-OHDA lesions were transplanted with mouse VM alone or combined with one of the liposomal immunosuppressive preparations. Good survival of transplants was

found in immunosuppressed groups 6 weeks after transplantation, in particular in those receiving liposomal TAC with transplanted cells no side effects were reported. This preparation of TAC was designed to reduce adverse effects of the drug; since the liposomes are taken up mainly by the reticuloendothelial system, TAC does not distribute well to the CNS and kidney therefore reducing neuro- and nephrotoxicity (Alemdar *et al.* 2007). A more recent study has demonstrated survival of human xenografts in rat hosts immunosuppressed with subcutaneously implanted slow release TAC pellets, showing a reduction in immune infiltration and survival up to 3 months post-transplantation (Sevc *et al.* 2013). These are promising methods which require further validation and assessment prior to implementation in transplantation studies.

1.3.2 Costimulatory pathway blocking

As previously described in section 1.2.2, two signals are required to initiate an immune response to transplants. First the formation of an MHC-TCR complex, and second the interaction of costimulatory molecules with their ligands. A body of research aims to determine whether manipulation of this interaction via costimulatory molecule blocking can produce tolerance to transplants. As discussed, ligation of costimulatory pairs can lead to a stimulatory or inhibitory response, therefore this must be considered when blocking for example the CD28/B7 pathway, since interfering with CTLA-4/B7 interactions can interfere with tolerance mechanisms (Li *et al.* 2009). However a number of experiments have reported successful tolerance to transplants following blocking of costimulatory molecules. Injection of allogeneic donor splenocytes into mouse hosts with treatment with an antibody to CD40L was shown to promote survival for at least 100 days without further immunosuppression, even though *in vitro* allo-responsiveness to donor cells was demonstrated. The authors suggested a state of “split tolerance” in mouse hosts (Markees *et al.* 1997). Additional studies have shown successful transplant tolerance following administration of a fusion protein containing the extracellular domain of CTLA-4 and the Fc portion of IgG; CTLA-4-Ig, designed to block CD28/B7 interactions by binding B7 molecules. This takes advantage of the fact that B7 molecules preferentially bind with CTLA-4. Studies have shown blocking of T cell proliferation in response to alloantigen stimulation *in vitro*, as well as the induction

of T cell anergy in response to treatment with CTLA-4-Ig (Li *et al.* 2009). Administration *in vivo* has been shown to induce donor-specific transplant tolerance to cardiac allografts, which could survive for at least 100 days after transplantation (Pearson *et al.* 1994). Optimal results have been reported when blocking both of these costimulatory pathways simultaneously (Larsen *et al.* 1996)

A recent study aimed to use these methods with a triple costimulatory blocking therapy to induce tolerance to ESC and iPSC derived transplants (Pearl *et al.* 2011). Treatment comprised administration of three costimulatory receptor blocking antibodies (CTLA4-Ig, Anti-LFA-1 and anti-CD40L) at 0, 2, 4 and 6 days post-transplantation. The method was shown to successfully prevent rejection of xenogeneic human ESCs and iPSCs in adult murine hosts as compared with those which were untreated or treated with tacrolimus and sirolimus (a rapamycin inhibitor). Mouse hosts were shown to be tolerant to donor cells, exhibiting T cell anergy and no detrimental effects on the hosts' immunity to other cell types were observed (Pearl *et al.* 2011). This could offer a potential method of avoiding immune rejection; however survival so far has only been demonstrated up to 8 weeks post transplantation.

1.3.3 Transplantation into neonatal hosts

In rodents, the early neonatal period is a time of immaturity in the immune system, when few or no mature T cells are circulating (Kingsley *et al.* 2007). During this time it is known that neural transplants in rats, including xenotransplants of mouse or human tissue, can survive long term in the neonatal brain (Englund *et al.* 2002; Lund *et al.* 1987). Lund *et al* (1987) found that both mouse and rat retina transplanted into the neonatal rat brain survived up to a year after transplantation. However transplants in hosts over 8 days of age were rejected (Lund *et al.* 1987), demonstrating that the survival of neonatal transplants is restricted to the early neonatal period. Additionally the survival of these grafts has been shown to be unstable, without the induction of tolerance, since peripheral challenges including skin grafts and damage to the BBB result in rejection of the established xenograft (Pollack and Lund 1990). Despite this, it still provides a useful model with which to test donor cells, since long term survival allows assessment of the safety and differentiation of human donor cells *in vivo* (Kallur *et al.* 2006; Kallur *et al.* 2011;

Zietlow *et al.* 2012). Interestingly, it has been recently shown that this privilege afforded to xenografts delivered to the neonatal rat immune system is not replicated in mice (Mattis *et al.* 2014). The authors found that transplants of human iPSC derived NPCs and human foetal NPCs transplanted into neonatal mice were rapidly rejected, as compared to those transplanted into neonatal non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (Mattis *et al.* 2014). It was also noted that the only successful mouse neonatal xenotransplants to have been reported were carried out in an inbred shiverer model (Windrem 2002), but that all follow up studies were subsequently carried out in shiverer mice crossed with *rag2* knockout (KO) mice (Wang *et al.* 2013a).

Although this method can be used with some success in the safety and integration testing of human donor cells, there is a limit to how well the function can be tested. For example, if transplants are carried out neonatally, it does not allow the investigation of the improvement of function following a lesion. Additionally the implantation of cells into the neonatal brain cannot be considered to be comparable to that of the adult brain, since the neonatal brain provides a more permissive environment with the presence of more developmental signals. Thus it is not a good test of transplantation into the adult brain which is the usual therapeutic situation. Therefore, although providing some benefits, the neonatal transplant model is not feasible for the purpose of full pre-clinical assessment of human donor cells.

1.3.4 Immune compromised hosts

As mentioned previously immune compromised rats or mice may also be used as transplant hosts, preventing an immune reaction to xenografts. Numerous models exist in each species for assessment of the differentiation and integration of donor cells in the adult host brain. A number of groups have used athymic nude rats to investigate the phenotype of hPF and hESC derived cells *in vivo* (Hurelbrink *et al.* 2002; Hurelbrink and Barker 2005; Nasonkin *et al.* 2009). The ability to assess the development of grafted cells long term *in vivo* is important to characterise changes which may not be detected with only short term survival as provided by immunosuppressant drug treatments. For example, in characterising the development *in vivo* of transplanted human ESC (hESC) derived striatal progenitors differentiated according to their protocol for directing ESCs to a MSN phenotype, Aubry and

colleagues transplanted into immunocompetent hosts to study short term graft survival, and nude rats for long term. Although the short term survival provided some positive results regarding their protocol, transplants into the nude rats showed overgrowth by around 2 months post-transplantation (Aubry *et al.* 2008). A number of models exist in mice, including SCID mice, *Rag1* or *Rag2* KO mice; with no mature B and T lymphocytes (Mombaerts *et al.* 1992; Shinkai *et al.* 1992). These rodents have been used for testing transplants of hESC derived striatal neurons (Ma *et al.* 2012), human glial restricted precursor cells (Janowski *et al.* 2012), and human iPSC derived oligodendrocyte progenitor cells (Wang *et al.* 2013a)

However, to assess the functionality of grafts it is necessary to perform behavioural tests on the animals to determine whether improvements may be observed. As immune compromised hosts are so susceptible to infection, the opportunity to test them behaviourally is unfortunately limited, thus restricting findings from these studies to the development and integration of grafts without the ability to correlate these findings with a functional read-out.

Other treatments, including the potential use of monoclonal antibodies to block T cell responses (anti-CD4/CD8), and blocking of costimulatory pathways offer the potential to induce tolerance to transplants (Robertson *et al.* 2007). The induction of tolerance by taking advantage of mechanisms discussed in section 1.4 is an additional method for promoting survival of transplants, for example through the induction of Treg differentiation, or the manipulation of DCs to induce tolerance (reviewed in Boyd and Fairchild (2010)). These offer potential methods to avoid rejection of donor cells both in pre-clinical or clinical transplant contexts, however they require further optimisation prior to successful application. Although the methods discussed here provide a range of different immunosuppression approaches to promote survival of human xenografts in rodent models of disease, none can provide an effective long term solution with which to test the full functional efficacy of donor cells. Either the host environment is inappropriate, or the model not robust enough for behavioural assessment. Drug treatments result in severe side effects, not allowing sufficient time for functional analysis, and transplant survival can be variable. Thus an alternative model is required for adequate testing of human donor cells for transplantation.

1.4 Immune tolerance

One approach, shown previously to successfully promote survival of skin allografts without immune suppression in adult rodents, is the induction of neonatal tolerance to allogeneic tissue after birth, when the immune system is still immature (Billingham and Brent 1956; Billingham *et al.* 1953). It is thought to be based on similar mechanisms to those governing self-tolerance in the normal immune system. This is achieved through self-antigen recognition by T cells, dependent on the development of immature thymocytes and their TCRs which in turn will be governed by the selection of self-antigens present during development of the thymus (Kyewski and Derbinski 2004). Self-tolerance is mediated by two coordinated mechanisms; 'central tolerance' in the thymus, and 'peripheral tolerance' to allow removal of those cells in the periphery which may have escaped selection in the thymus. This section discusses the mechanisms of natural "self" tolerance, and how this relates to the induction of neonatal tolerance to allografts.

1.4.1 Central tolerance

Hematopoietic stem cells (HSCs) in the bone marrow can differentiate into red blood cells, platelets and cells of the innate and adaptive immune system, including T cells. Once immature T cells have developed from HSCs they migrate to the thymus to undergo maturation where they are subjected to the selection which allows the immune system to recognise and remember pathogens as well as to remain tolerant to "self" antigens, preventing autoimmunity. On arrival at the thymus through the corticomedullary junction, T cells are 'double negative' (DN) lacking expression of both CD4 and CD8 (Starr *et al.* 2003). In the thymic cortex thymocytes become committed to either an αβ or γδ lineage dependent on levels of Notch activity (Washburn *et al.* 1997). Those which become αβ T cells must undergo successful rearrangement of TCR β chain, then α chain followed by the upregulation of both CD4 and CD8, moving them to the double positive (DP) stage.

As the development of T lymphocytes is dependent on random gene rearrangement of T cell receptor loci this ensures recognition of a diverse range of antigens which may be encountered, however it also means that some cells will bear self-reactive TCR specificities. In order to avoid autoimmunity a selection process is

required to remove auto-reactive cells prior to maturation and migration to the periphery. Cells are first subjected to positive selection dependent on the binding of their TCRs to MHC class I and II complexes presented on cortical thymic epithelial cells (cTECs) (von Boehmer 1994). DP T cells are positively selected if their TCRs interact with self MHC molecules on cortical epithelial cells. They are programmed for apoptosis unless signalling is received via TCR binding to self MHC, therefore those which show no self-recognition undergo death by neglect (Starr *et al.* 2003). αβ T cells respond to antigens via peptide binding to MHC class I or II molecules expressed on the cells surface. MHC class I molecules detect and present endogenous peptides, whereas MHC Class II molecules target exogenous peptides (Germain 2002). Antigen recognition by αβ T cells is therefore dependent on surface expression of CD4 or CD8. This surface phenotype is determined by the interaction between the cells TCR and the MHC complex; CD4 expressing T cells bind MHC class II and become helper or regulatory T cells and $CD8^+$ cytotoxic T cells bind MHC class I (Palmer 2003; Swain 1983).

After positive selection and commitment to either a $CD4^+$ or $CD8^+$ single positive (SP) lineage, thymocytes migrate to the thymic medulla for negative selection where those cells whose TCRs show high avidity binding with self MHC class I or II complexes undergo apoptosis (Metzger and Anderson 2011). This reduces the number of self-reactive T cells which can mature and move to the periphery and therefore the potential for an autoimmune response to be initiated. APCs are required for negative selection, therefore it is suggested to take place in the medulla due to its large population of APCs; medullary thymic epithelial cells (mTECs) and DCS, (Sprent 1995; Webb and Sprent 1990). **Figure 1.4** shows a simplified diagram of T-cell development in the thymus (Germain 2002).

The deletion of thymocytes which react to self antigens, requires the representation of self antigens within the thymus (Sospedra *et al.* 1998). Mature mTECs express CD80 and MHC-II, a subset of which express the transcription factor autoimmune regulator (Aire) for approximately one week prior to apoptosis (Gray *et al.* 2007). Aire deficiency results in autoimmune disorders (Capalbo *et al.* 2012), as found in Aire-deficient mice, which display lymphocyte infiltration of multiple organs due to severe autoimmunity (Anderson *et al.* 2002). The expression

of Aire promotes the ectopic expression of tissue restricted self-antigens (TRA) to developing T cells in the thymus, establishing central T cell tolerance. These antigens are usually exclusively expressed in organ specific cell types, therefore this is referred to as promiscuous gene expression (Holländer and Peterson 2009); with a single mTEC capable of representing TRAs from a number of different peripheral tissues (Derbinski *et al.* 2001). Promiscuous gene expression of TRA on mTECs, and cross presentation of these antigens by DCs which capture and present TRA secreted by mTECs inform negative selection of T cells which are TRA-reactive (Gallegos and Bevan 2006). Although promoting the expression of peripheral TRAs in the thymus, mTECs are unlikely to express all self antigens present within the body sufficient for deletion of all self-reactive T cells. It has additionally been shown that populations of DCs can migrate to the thymus, transporting peripheral antigens for promoting central tolerance (Bonasio *et al.* 2006).

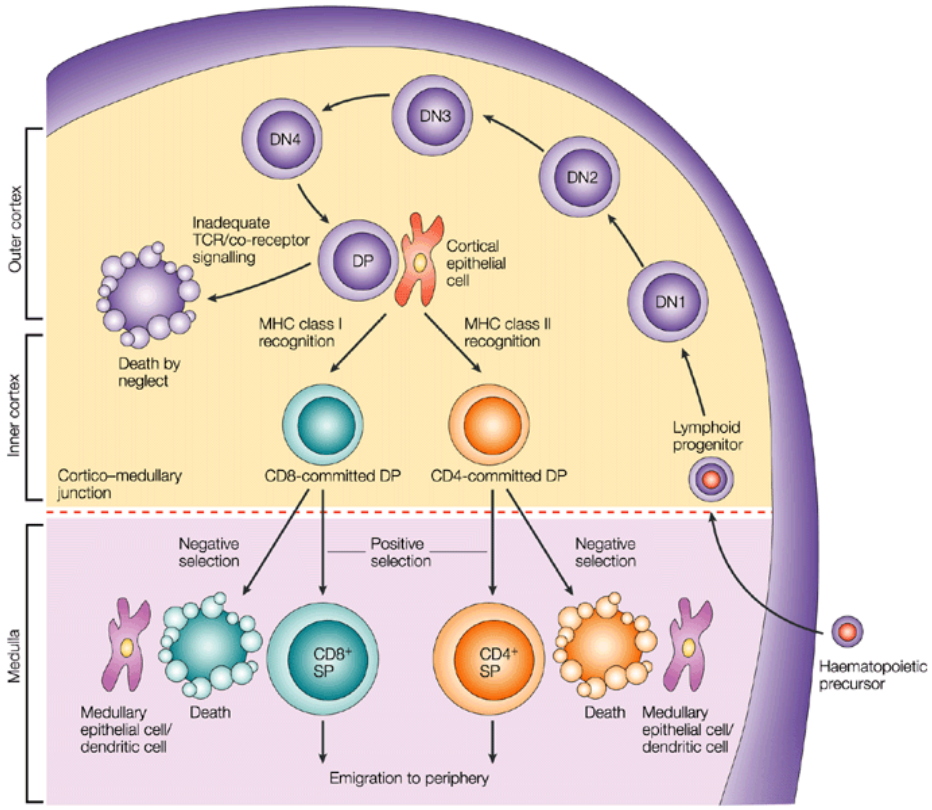

Figure 1.4 T cell development in the thymus (reproduced from Germain (2002))

1.4.2 Peripheral tolerance

The maintenance of immune system homeostasis and tolerance to self antigens also relies on the control of self-reactive T cells by regulatory T cells (Tregs) (Sakaguchi *et al.* 2008). Natural Tregs develop in the thymus in response to signalling via TCRs, as with positive and negative selection, though the commitment to a Treg lineage is the result of TCRs which display an avidity range between that required for positive and negative selection (Metzger and Anderson 2011). Self-reactive thymocytes which escape negative selection are therefore directed towards a Treg lineage (Kim *et al.* 2007). Additionally, a role for Aire has been implicated in the development of Tregs via antigen presentation from $Aire^{+}$ mTECs (Hinterberger *et al.* 2010). The development of natural Tregs is characterised by expression of Foxp3, and this is responsible for the dominant form of T cell tolerance both in the thymus and the periphery. The absence of Tregs removes control of autoreactive T cells in the periphery resulting in lethal autoimmunity (Kim *et al.* 2007).

An additional role for Aire in the promotion of peripheral tolerance has been proposed with the discovery of the expression of Aire in peripheral lymphoid organs; spleen and lymph nodes in mice (Zuklys *et al.* 2000), and just the lymph nodes in humans (Gardner *et al.* 2008). It is thought that extrathymic Aire expressing cells (eTACs) have the ability to tolerise peripheral T cells via deletional tolerance, comparable to negative selection in the thymus, with the presentation of a distinct set of TRAs from those expressed by mTECs in the thymus. This comprises a complementary role in tolerance for Aire in the periphery and the thymus (Gardner *et al.* 2008). Thus mechanisms in the periphery exist to suppress the activity of autoreactive T cells which may have escaped negative selection in the thymus, although the maintenance of this peripheral tolerance is likely to be controlled by central mechanisms.

1.4.3 Neonatal tolerance

The induction of tolerance to allografts in mice has been described for decades with the finding that an injection of donor cells into neonatal mouse hosts can prevent subsequent rejection of a skin graft from donors of the same strain (Billingham and Brent 1956; Billingham *et al.* 1953; Medawar 1948). Previous work has subsequently shown the successful induction of tolerance to allogeneic skin grafts following injections of spleen (Adkins *et al.* 2004; Ridge *et al.* 1996), liver (West *et al.* 1994) or bone marrow cells (Modigliani *et al.* 1997). Hosts in these studies were demonstrated to be tolerant to allogeneic donor cells by the survival of donor skin grafts or cardiac allografts and a lack of host cytotoxic T lymphocyte (CTL) responses to donor spleen cells *in vitro* (Adkins *et al.* 2004; Modigliani *et al.* 1997; West *et al.* 1994).

Some mechanisms for the phenomenon of neonatal tolerance following injections of allogeneic cells during the neonatal period have been proposed, although a definitive conclusion has not been reached. It has been suggested that for tolerance induction, as with the natural development of "self" tolerance, the persistent presence of donor or "self" cells are required during the development of the immature immune system. Passive models suggest that neonatal tolerance occurs through negative selection of self-reactive T cells, as is the case in the natural development of self-tolerance. The presentation of donor antigens in the thymus, along with self antigens therefore allows donor reactive thymocytes to also be negatively selected. As few mature T cells exist neonatally, donor cells are able to reach the thymus to promote this deletion of donor reactive T cells (Morrissey *et al.* 1983; Ridge *et al.* 1996). Active models suggest that newly developing T cells present in the neonatal host generate mainly T helper cell 2 (TH2) responses, protecting from self recognition and also reducing recognition of donor cells present at this stage (Bandeira *et al.* 1989).

However, these models imply an inability of the neonatal immune system to mount an immune response. This does not seem logical, since the neonate would therefore have no natural defence against invading pathogens, which has been highlighted by Matzinger and colleagues. They have highlighted the ability of neonates to become immunised rather than tolerised to neonatally presented viruses

(Miller *et al.* 1994) which does not agree with these models (Ridge *et al.* 1996). They therefore propose a third model, the "Danger" model suggesting that it is not the host population of T cells which governs the induction of tolerance, rather it is what constitutes the population of injected donor cells which determines tolerance or immunity (Matzinger 1994; Ridge *et al.* 1996). When the population of cells used for neonatal administration does not include a large proportion of mature APCs, then a lack of co-stimulation of host T cells occurs resulting in a tolerising response. Conversely, if a large population of active APCs such as DCs are present in the injected suspension, an immunising effect will occur. The authors found exactly these responses when injecting spleen cells or isolated DCs (Ridge *et al.* 1996). Tolerance induction was found when injecting a large number of spleen cells neonatally, whereas injecting an enriched population of DCs primed T lymphocytes for attacking donor cells. Much variation has been found in this area, with some disagreement for this proposal from a study showing that neither donor T nor B cells are required for the induction of neonatal tolerance, since successful tolerance has been induced from injection of spleen and bone marrow cell suspensions from *rag1*$^{-/-}$ mice which contain neither of these populations, but may contain professional APCs (Chan *et al.* 2007; Modigliani *et al.* 1997).

To successfully induce neonatal tolerance in mice has required the injection of large numbers of spleen cells, as well as other cell types (e.g. $1.5x10^7$ – $1x10^8$) (Ando *et al.* 1991; Peiguo *et al.* 2012). Studies suggest that the successful induction of tolerance in neonatal hosts is dose dependent with the injection of larger cell numbers increasing the potential of inducing tolerance (Peiguo *et al.* 2012), and the injection of low numbers of cells resulting in the opposite effect; priming of CTL (Adkins *et al.* 2004). This difference is thought to be due to the ratio between the number of injected donor cells and the number of circulating potentially responsive T cells within the host. It has been concluded that in order for successful induction of neonatal tolerance, all potentially responsive circulating T cells must be inactivated (Ridge *et al.* 1996).

This phenomena has been demonstrated numerous times in tolerance to mouse allografts, though less success has been observed in the case of xenografts (Shen *et al.* 1996). It is possible that species differences in TCR recognition of xenogeneic

donor MHC molecules prevents the induction of neonatal tolerance, although this has been disputed by Borenstein *et al.* (2004). By taking advantage of a Tg mouse model expressing the human MHC class I allele (HLA)-B7, the authors found that an i.v. injection of $1.5x10^7$ spleen cells from Tg mouse donors could induce tolerance in B6 mice, sufficient to promote survival of skin grafts from Tg HLA-B7 mouse donors. This demonstrates that the host B6 immune system was capable of recognising donor xenogeneic MHC molecules (Borenstein *et al.* 2004) and that the inability to induce tolerance to xenoantigens must be due to MHC-independent interactions with the recipient immune system. By staining of host spleen cells for HLA-B7, the authors could assess the level of chimerism in host mice. Low levels of HLA-B7 staining were found in peripheral lymphoid organs suggesting engraftment of donor cells into recipient bone marrow and implying a correlation between chimerism and the induction of tolerance. Successful integration of donor cells within the host, and the subsequent induction of chimerism has been frequently suggested as a requirement for, if not at least to strongly correlate with successful neonatal tolerance in mice (Borenstein *et al.* 2004; Chan *et al.* 2007).

1.5 Neonatal desensitisation

One of the aims of the work in this lab group is the testing of potential human donor cells for transplantation in neurological disease. As this requires the xenotransplantation of human cells into rodent hosts, potentially for long periods of time, one problem that is regularly faced is that of adequate and tolerable immune suppression. Problems with the current available methods have already been discussed, highlighting the need for potential new models to achieve this goal. The knowledge that transplantation of xenogeneic tissue into the neonatal rat brain can survive for at least a year after transplantation without additional immune suppression (Englund *et al.* 2002; Lund *et al.* 1987) has been exploited in the design of a method of avoiding conventional immune suppression. As the developing immune system in the rat is known to provide an environment permissive of transplant survival, work was undertaken in this lab to determine whether the survival of a second transplant in adulthood may be promoted in this way. This was successfully carried out with the intraperitoneal (i.p.) administration of a suspension of human foetal cortex (hCTX) derived donor cells in neonatal rats, followed by a subsequent neural transplant of human whole ganglionic eminence (WGE) which was found to survive without the need for further immunosuppression (**Figure 1.5**).

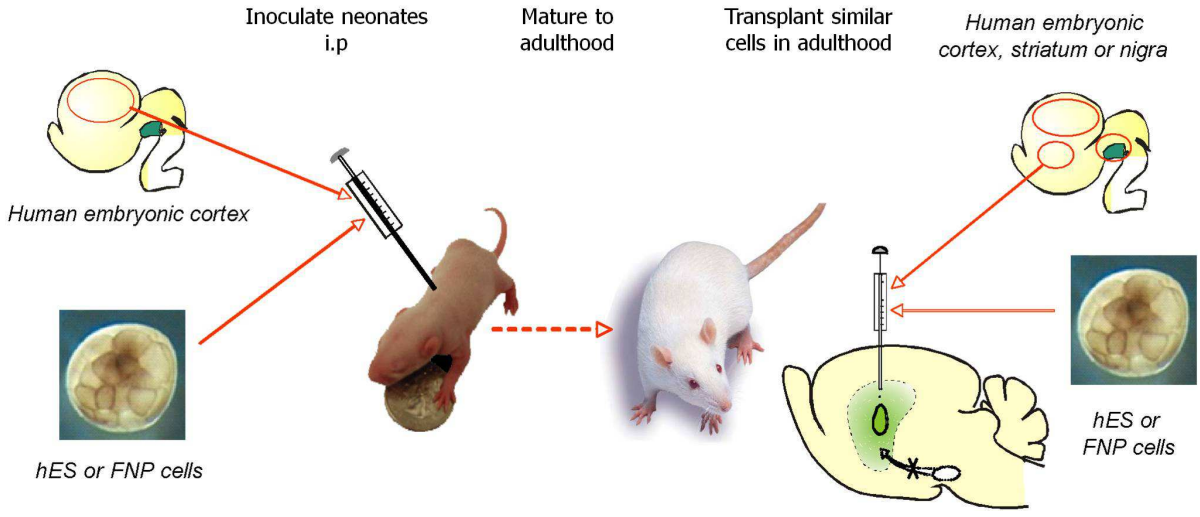

Figure 1.5 Schematic representation of the induction of neonatal desensitisation (courtesy of Kelly, CM).

This method was subsequently further developed to determine the characteristics of neonatal desensitisation in the rat (Kelly *et al.* 2009b). Intra-peritoneal (i.p.) injections of suspensions of human derived cells in neonatal Sprague Dawley (SD) rats were followed by adult transplants of these cell types. Survival of human cells was demonstrated up to 40 weeks after transplantation, and graft morphology was shown to be comparable to that found in immune suppressed animals. Side effects in CsA treated animals meant survival could not be assessed beyond 12-16 weeks as animals had to be culled according to animal licensing regulations. Transplants of human primary foetal neural tissue, foetal brain derived neural precursors and embryonic stem cell derived neural precursor cells were all shown to survive as long as, or longer than transplants into CsA treated animals following desensitisation with the relevant tissue (**Table 1.3**). Successful desensitisation could only be shown with inoculation of host animals up to postnatal day (P)5; those which received cells at P10 or in adulthood could not support the survival of a subsequent neural transplant. Additionally it was found that inoculation with dead cells was not sufficient for desensitisation of hosts (Kelly *et al.* 2009b).

It is possible that, by desensitising animals neonatally (referred to as 'tolerising'); tolerance has been induced to donor tissue in these host animals. Although true tolerance in hosts has not been identified, the method and graft survival observed is comparable to that described historically in studies of allogeneic skin graft tolerance induced neonatally by injection of cell suspensions of donor origin (Billingham and Brent 1956; Billingham *et al.* 1953) as discussed previously. However, since tissue used for desensitisation is from a different foetal donor to that used to transplant, it seems illogical that desensitisation represents tolerance to human tissue, particularly since no control has been made for matching of HLA haplotypes. The induction of neonatal tolerance seems to require the persistent presence of donor antigen during the neonatal period, at a stage of immaturity in the immune system before mature T cells migrate out from the thymus (Kingsley *et al.* 2007). This may result from the presence of viable cells with the potential for continued growth, or repeated injections of antigen in this period. This may then prevent the development of mature T cells which react with donor antigen, as in the case of the induction and maintenance of "self" tolerance. Neonatal tolerance induction in mice has commonly used spleen (Adkins *et al.* 2004; Ridge *et al.* 1996),

liver (West *et al.* 1994), or bone marrow cells (Modigliani *et al.* 1997) to provide this continuing source of donor antigen. It remains unclear whether human neural cells injected into SD rat neonates in previous experiments have resulted in the same "tolerance" as described in the neonatal tolerance field (Kelly *et al.* 2009b). Since human donor cells are derived from the developing brain, the potential for viability and proliferation exists in combination with the immaturity of the rodent immune system preventing their rapid rejection after injection. Additionally, as the rat immune system remains immature until 8-10 days after birth, an injection of cells at this time period would be protected from immediate rejection by the host immune system

This method has the potential to allow the preclinical testing of grafts from different cell sources in animal models of disease, avoiding the issues associated with traditional immune suppression regimes. However a number of findings need to be resolved to support its routine use. Although it has been shown that the presence of viable cells during the early neonatal period is required to induce desensitisation, the mechanisms behind successful desensitisation of neonates remain unclear. It has not been determined whether tolerance has been induced to donor cells or whether desensitisation is only sufficient to prevent initial graft rejection, and the host may be capable of mounting an immune response to donor cells when recognised, for example in the case of a peripheral challenge, or damage to the BBB as is the case for transplants in neonatal hosts (Pollack and Lund 1990). Additionally, for routine long term use of the method in pre-clinical tests of potential human donor cells, validation is required to determine the optimum protocol for successful promotion of graft survival. The method has also only been demonstrated as yet in the SD rat with transplants to the intact striatum; ideally desensitisation could be applicable in other host strains, with various donor cells such as ESCs and iPSCs, as well as in other host species including mice. The ability to test human donor cells in mice allows testing in transgenic models of disease, determining the effect of the host environment on transplanted cells.

Neonatal Desensitisation	Adult graft	Tissue concordance	Survival after transplant (weeks)	Number of grafts surviving (%)
None	hCTX	-	12	0/9 (0%)
None	hWGE	-	2	0/7 (0%)
CsA	hWGE	-	12	7/9 (77%)
CsA	hFNP	-	12	6/8 (75%)
mWGE	mWGE	Concordant	10	11/15 (73%)
mFNP	mFNP	Concordant	10	11/13 (85%)
hCTX	hCTX	Concordant	40	48/55 (87%)
hWGE	hWGE	Concordant	25	10/15 (66%)
hFNP	hFNP	Concordant	12	10/13 (77%)
hES-N	hES-N	Concordant	12	9/12 (75%)
hFNP	hCTX	Discordant	12	11/13 (85%)
hCTX	hFNP	Discordant	12	11/14 (79%)
hLiver	hCTX	Discordant	12	8/13 (62%)
hCTX	hWGE	Discordant	10	19/23 (83%)

Table 1.3 Graft survival in different desensitisation protocols, adapted from Kelly *et al.* (2009b). Hosts in all groups were SD rats. Data shows no surviving grafts in untreated hosts, with improved survival in hosts treated with CsA daily beginning from one day prior to transplantation. Greatest graft survival is seen in hosts desensitised neonatally with hCTX and transplanted with hCTX. *hCTX = human primary foetal cortex, hWGE = human whole ganglionic eminence, hFNP = human foetal neural precursors derived from human foetal cortical tissue, mWGE = mouse whole ganglionic eminence, mFNP = mouse foetal neural precursors from mouse foetal cortical tissue, hES-N = human embryonic stem cell derived neural precursors, hLiver = human liver*

To date a number of groups have shown interest in the use of the method for transplantation of a number of different human cell types in a variety of animal models. Some success has been found in inducing desensitisation in rats to human cells, including hESC-MSC transplanted following induced knee cartilage defects (Zhang *et al.* 2013). Host rats were desensitised neonatally with $1x10^5$ hESC-MSC prior to adult transplants of a collagen bilayer scaffold seeded with hESC-MSC. The authors reported survival of transplants in desensitised hosts up to 8 weeks post-transplantation, with rejection of cells in hosts which were not desensitised neonatally. In an assessment of the immune response one week after transplantation, a reduction in $CD4^+$ T cells was found in desensitised hosts, thus the authors reported successful desensitisation to hESC-MSC (Zhang *et al.* 2013). In addition to this, Singhal *et al* aimed to investigate the potential of human Müller glia stem cells to differentiate into retinal ganglion cells (RGC) in a rat model of RGC depletion (Singhal *et al.* 2012). In this study, Lister Hooded rat hosts were desensitised neonatally with an injection of $1x10^5$ human Müller stem cells prior to transplantation at 3-4 weeks of age with undifferentiated stem cells or RGC precursors. Survival was demonstrated 4 weeks after transplantation, however the authors also administered oral CsA, prednisolone and azathioprine to ensure survival of transplanted cells (Singhal *et al.* 2012).

Other groups, however, have reported less success with the method in experiments with different types of donor cells and different host species. Jablonska *et al* compared three methods of immunosuppression, including neonatal desensitisation, to prevent rejection of human cord blood derived neural stem cells (HUCB-NSC) for transplantation into infarcted rats (Jablonska *et al.* 2013). Neonatal desensitisation with $1x10^5$ HUCB-NSC failed to promote survival of adult transplants of these cells beyond 21 days post-transplantation. However; the authors also failed to prevent rejection of these cells with either daily CsA injections at a standard dose (10mg/kg), or a triple-immunosuppression protocol with daily CsA, azathioprine and methylprednisolone, therefore it cannot be assumed that graft loss in this case was due to ineffectiveness of the neonatal desensitisation protocol and strongly suggests a general problem with donor cell survival (Jablonska *et al.* 2013). The same group and collaborators attempted desensitisation in mice to human glial restricted precursor cells (hGRP) and in rats to HUCB-NSC with poor survival

observed in both cases, as compared to surviving transplants of both cell types demonstrated in *Rag2*$^{-/-}$ mice, thus reporting that the method could not be reliably replicated (Janowski *et al.* 2012). Although unclear why desensitisation in rats was unsuccessful considering previous findings from this and other research groups, it is possible that successful desensitisation may depend on the host model and the type of cells used (Kelly *et al.* 2009b; Kelly *et al.* 2012).

More consistent issues have been recently highlighted with attempts to induce neonatal desensitisation to human donor cells in mouse hosts. In addition to the unsuccessful desensitisation in mouse hosts reported by Janowski *et al.* (2012), a recent paper systematically investigated the desensitisation protocol in three different strains of mice with three different types of human stem cells and found no successful promotion of xenograft survival using the method (Mattis *et al.* 2014). Survival was demonstrated in immune suppressed hosts up to 6 weeks after transplantation, although grafts were significantly smaller than earlier time-points. However, transplants in desensitised hosts were consistently rejected by 2 weeks post-transplantation along with a strong host microglial response. Interestingly the authors also investigated the survival of human iPSC derived neural precursor cells (iPSC-NPCs) and human foetal neural precursors (hFNPs) transplanted to the neonatal mouse striatum, finding no survival past 21 days post-transplantation, in contrast to surviving transplants demonstrated in immunodeficient NOD/SCID mouse hosts (Mattis *et al.* 2014). This is suggestive of a difference in both the neonatal and adult mouse immune system compared to that of the rat, in which neonatal desensitisation to human donor cells has been demonstrated successfully (Kelly *et al.* 2009b; Singhal *et al.* 2012; Zhang *et al.* 2013) as well as consistent survival of neonatal xenotransplants (Hurelbrink *et al.* 2002; Hurelbrink and Barker 2005; Lund *et al.* 1987; Zietlow *et al.* 2012).

1.6 Aims

The overall aims of the experiments presented in this thesis are to further characterise and validate the desensitisation method described above. Although neonatal desensitisation has been demonstrated by both our lab group and others to be a successful method for evading immunological rejection of neural xenografts, the parameters of the method have not yet been defined and much variability has been observed in experiments using different species and different donor cell types. To date this group has only found confirmation of successful desensitisation in striatal and VM transplants of human neural tissue into Sprague Dawley (SD) rats which had been desensitised with tissue of the same type. Further investigation into the parameters and limitations of the method will allow its use in grafting studies in other species and strains to test the potential of a range of donor cells. In addition, studying the mechanisms of the method will provide valuable information on the induction of transplant tolerance relevant across a number of fields. All experiments have been carried out using the QA lesion model of HD, providing a suitable model of cell loss in which to transplant human donor cells and investigate survival without the complications of interaction with potential immune system differences in Tg models of HD.

Primarily, the initial objective is to validate the method in the mouse using similar grafting protocols to those used previously in the rat (Kelly *et al.* 2009b). As an extension of this, the optimum numbers of cells required to inject neonatally to allow survival of xenografts must be investigated, as this may differ from species to species. Secondly, as the method has already been shown to be successful in the rat, a number of parameters are also examined in this species to determine what tissue type may be used to inject at birth and allow survival of transplanted neural tissue, for example using non-neural tissue to induce tolerance to a neural graft, and whether the technique may be applied successfully across other strains of rat. Finally, in addition to studying the parameters of the method, I also intend to investigate the underlying mechanisms of the method.

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Chapter 2

Materials and Methods

2.1 *In vitro* methods

All reagents and suppliers are listed in **Appendix 1**. Recipes for solutions and protocols are listed in **Appendix 2**.

2.1.1 Collection and dissection of primary human tissue

Ethical approval was granted for these studies by the Bro Taf Local Research Ethics Committee and more recently the Cardiff and Vale University Health Board. Human cortical tissue (ranging from 7-12 weeks gestation) was collected from the donation of the products of elective terminations of pregnancy (ToP). All tissue was donated through SWIFT, funded by the Medical Research Council (MRC) and a Welsh government grant. Consent for donation was given only after the completion of procedures for approval, consent and scheduling of ToP. Tissue was collected from medical termination of pregnancy (mToP) procedures (Kelly *et al.* 2011) and was transported to the laboratory in sterile hibernation medium (Hibernate E, *Gibco, Paisley, UK*). Ultrasound prior to the ToP was used to stage embryos; this was then confirmed at dissection by measurement of crown rump length (CRL), as shown in **Appendix 3**. After careful removal of the brain, the relevant tissue was dissected and prepared as a dissociated cell suspension[1].

2.1.2 Collection and dissection of primary mouse tissue

All animal experiments and surgical procedures were conducted under the UK Animals (Scientific Procedures) Act 1986 as amended, and subject to local ethical

[1] Tissue collection and dissection for initial experiments was carried out by other members of the BRG (Claire Kelly, Sophie Precious, Rike Zietlow and Eduardo Torres) until the relevant licenses and training were completed and I was able to collect and dissect subsequent samples.

review and relevant personal, project and institutional licenses. All animals were housed in a natural light-dark cycle with *ad libitum* access to food and water. Pregnant CD-1 mice were purchased at late gestation (*Harlan, Bicester, UK*), killed via cervical dislocation and embryos collected at the specified donor age (E12 or E14) in Hanks' Balanced Salt Solution (HBSS, *Gibco*). Brains were removed and striatal tissue dissected as shown in **Figure 2.1** using a dissecting microscope in a laminar flow hood.

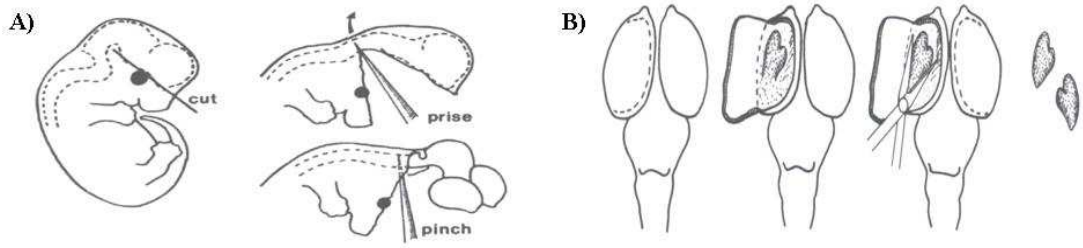


Figure 2.1 A) Removal of brain from embryos. A single vertical cut is made just above the eye at the base of the brain back into the ventral mesencephalic flexure. Fine forceps are gently inserted under the skin to remove the overlying skin and meninges leaving the brain free to be pulled away. B) Striatum (WGE) removal. The brain is positioned on its ventral surface with the dorsal cortex facing upwards. A longitudinal cut is made through the medial cortex which once folded over exposes the striatum on the floor of the lateral ventricle. Iridectomy scissors are used to remove the striatum from both hemispheres. Cortical tissue is collected by carefully removing the overlying meninges (Adapted from Dunnett, 1996).

2.1.3 Preparation of cell suspensions

Human or mouse tissue was incubated for 20 minutes at 37^{o}C in bovine trypsin (*Worthington, New Jersey, USA*), after which DNase (*Sigma, Gillingham, UK*) and bovine trypsin inhibitor (*Sigma*) was added for a further 5 minutes. The tissue was washed in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, *Gibco*) then centrifuged for 3 minutes at 1000 rpm. Cells were re-suspended in DMEM/F12 and mechanically dissociated by trituration using a Gilson pipette with 200 µl tips. Cell numbers and viability were determined by trypan blue (0.4% trypan blue solution, Sigma) exclusion counting using a haemocytometer. Cell suspensions were only used if viability was found to be over 75% for neonatal injection and 80% for intracerebral transplantation. 10 µl of cell suspension was diluted with DMEM/F-12 and trypan blue, transferred to a haemocytometer with a glass cover-slip and viewed under the microscope. The number of living cells and total cells were counted in 5 squares of the haemocytometer to give an average cell

number per square. The number of cells per µl could then be calculated taking into account the dilution factor below:

Cells counted/squares counted x dilution factor x 10 = cells/µl

Following calculation, cells were re-suspended to the required concentration for desensitisation or transplantation

2.2 *In vivo* methods

2.2.1 Animal care, anaesthesia and immunosuppression

Pregnant dams (rat or mouse) were purchased (*Harlan, UK*) and housed individually until they gave birth with *ad libitum* access to food and water on a standard light-dark cycle. Pups were weaned at 28 days and mothers sacrificed or used as control host animals. Remaining control hosts were purchased as adults (~12 weeks of age). After weaning animals were then housed in same sex groups of 2-4 mice or rats per cage and experiments began in adults weighing 200-250 g for rats, or 20-25 g for mice.

All surgical procedures were performed under isoflurane anaesthesia induced in an induction chamber with 5% isoflurane in oxygen at 0.8 L/min. Anaesthesia was maintained by passive inhalation of isoflurane (1.5-2.5%) in a mixture of oxygen (0.8 L/min) and nitrous oxide (0.4 L/min). Animals recovered in a warm recovery chamber before being returned to their home cage and health and weights were monitored for two days following surgery.

For those animals receiving xenotransplants which were not tolerised, most received daily immunosuppression to allow graft survival. Daily i.p. injections of CsA (*Sandimmun, 10mg/kg; Novartis, Hampshire, UK*) were administered for the duration of the experiment, starting a day before transplantation.

2.2.2 Neonatal Desensitisation

Those pups to be desensitised were separated briefly from their mothers between post-natal day 0 (P0) and P4 to receive i.p. injections of 1 µl of cell suspension in sterile DMEM solution via a 1 µl handheld glass microsyringe. Cells were usually injected at a concentration of $1x10^5$/µl, though this varied in some experiments. Care was taken to avoid transfer of odours and potential rejection of pups by the mother, with all handling carried out wearing disposable nitrile gloves. Following injections, pups were returned immediately to the mother.

2.2.3 Quinolinic Acid Lesions

QA was dissolved in 0.1 M phosphate-buffer to make a 90 mM solution. Animals received a unilateral injection of QA to the right striatum. Prior to surgical procedures animals were administered sub-cutaneous injections into the scruff of meloxicam (Metacam) for analgesia; 5 µl for mice, and 30 µl for rats. The skull was then exposed; a small burr hole drilled above the right striatum and QA was injected via a cannula attached to a 10 µl Hamilton syringe driven by a mechanical pump.

For QA lesions in mice the coordinates for injection sites were: 0.8mm rostral to bregma (AP) -2.0 mm lateral to midline (L) and -3.0/-2.8 mm ventral from dura (V). 0.75 µl of 0.09 M QA was injected over 6 minutes, the needle was then left in place for 3 minutes to prevent reflux of toxin up the needle tract. The incision was sutured and animals were administered subcutaneous injections to the scruff of 0.5 ml saline glucose to prevent dehydration, and an intramuscular (i.m.) injection of 30 µl diazepam into the upper leg for sedation to prevent seizures following lesion.

Rats received 2 simultaneous injections of QA over 3 minutes, for 1.5 minutes each at 2 depths. Stereotaxic coordinates from bregma were +0.4/+1.0mm (AP) and -3.5/-2.8 mm (L) at a depth of -5.0 mm and -4.0 mm below dura. Following infusion the needle was left in place for 3 minutes before removal. After suturing, animals received a subcutaneous injection of 5ml saline glucose and 0.15 ml diazepam (i.m.) as before.

2.2.4 Striatal Transplants

For transplants into adult mouse brain, 2 µl of cell suspension ($1.25x10^5$ or $2.5x10^5$ cells/µl) were delivered at a rate of 1 µl for 1.5 minutes each at two depths using a Hamilton syringe. Grafts were injected into the lesioned or intact right striatum using coordinates +0.8 mm (AP), -2.0 mm (L) from bregma at -3.2 and -2.8 mm below dura. After grafting, the needle was left at the graft site for a further 3 minutes before being withdrawn. The incision was sutured and animals were administered subcutaneous injections of 0.5 ml saline glucose and 5 µl meloxicam.

For transplants into adult rat brain, 2µl of cell suspension ($2.5x10^5$ cells/µl) were delivered at a rate of 1 µl/minute with 1 minute each at two depths using a Hamilton

syringe. Grafts were injected into the lesioned or intact right striatum using coordinates +0.7 mm (AP) and -3.1 mm (L) from bregma at -4.5 mm and -3.5 mm below dura. The needle was left in place for 2 minutes before being withdrawn. The incision was sutured and animals were administered subcutaneous injections of 5 ml saline glucose and 30 µl meloxicam. For bilateral transplants, the coordinates above were used to inject into the right striatum, and for the left; -0.7 mm (AP) and +3.1 mm (L) from bregma at the same depths as above.

2.2.5 Perfusions and sectioning

Animals were terminally anaesthetised by i.p. administration of sodium pentobarbital (Euthatal) and transcardially perfused with a prewash solution (phosphate buffered saline (PBS) at pH 7.3) for 2 minutes followed by 1.5% or 4% paraformaldehyde (PFA) solution at pH 7.3 for 4 minutes. The brains were removed, post-fixed in 1.5% or 4% PFA overnight and transferred the following day to 25% sucrose in prewash solution until they sank.

Brains were sectioned coronally at 40 µm thickness using a freezing-stage microtome. Sections were stored either in 0.01% azide in Tris-buffered saline (TBS) in 96-well plates at 4^{o}C, or in anti-freeze at -20^{o}C.

2.2.6 Cresyl Violet staining

Brain sections (1 in 12 series) were mounted onto glass microscope slides double-subbed with 1% gelatin and allowed to dry overnight. Sections were soaked for 5 minutes each in increasing levels of alcohol from 70%, to 95% then 100% followed by 1:1 chloroform ethanol for 20 minutes. Slides were then put back through the decreasing alcohols for 5 minutes each from 100% to 95% to 70% followed by distilled water, before incubation in cresyl violet stain for 5 minutes and rinsing in water for 5 minutes. Stained sections were dehydrated in 70% and 95% alcohol, then destained in 2.5% acetic acid in 95% alcohol for 2-5 minutes until the desired level of staining was reached. Further dehydration was carried out in 95% and 100% alcohol, and then sections were cleared in xylene before coverslips were applied using DPX mountant.

2.2.7 Immunohistochemistry on free-floating tissue sections

1 in 12 series of brain sections were washed thoroughly in TBS (pH 7.4) quenched with 10% hydrogen peroxide and 10% methanol in distilled water for 5 minutes followed by three 5 minute washes in TBS. Sections were blocked for 1 hour in 3% appropriate normal serum in 0.2% Triton X-100 in TBS (TXTBS), then without washing, block was discarded and primary antibody was added at the appropriate concentration in 1% serum in TXTBS and incubated overnight at room temperature. Sections were washed 3 x 10 mins in TBS before addition of a biotinylated secondary antibody at 1:200 dilution in 1% serum for 2 hours. Primary and secondary antibodies and the relevant blocking sera are all lised in **Appendix** 4. Three washes in TBS removed the secondary antibody solution and strepdavidin ABC (A and B both at 1:200 dilution in 1% serum in TBS; prepared 30 minutes before use) was added for a further 2 hours. The sections were washed 3 x 10 mins in TBS, followed by 2 x 5 mins washes in 0.05 M tris non saline (TNS, pH 7.4) and positive staining was visualized using diaminobenzidine (DAB) at 0.5 mg/ml in fresh TNS with 12 µl hydrogen peroxide (brown). Following staining, sections were washed twice in TNS before mounting on gelatinized class microscope slides. Sections were allowed to dry, then dehydrated in increasing levels of alcohol, cleared in xylene and coverslips were mounted using DPX mountant.

2.2.8 Quantification and photomicroscopy of grafts

Visualisation of grafts was carried out under a Leica DRMBE light microscope. Images were captured using a Leica DFC420 camera and Leica Application Suite (LAS) image analysis software. Graft areas were measured by drawing round areas of HuNu+ or NeuN+ staining on Image J software, or using Visiopharm stereology software. Estimates of graft volume were calculated using the formula:

$(\sum a*M)/f$

a = area (μm^2), M = section thickness and f = frequency of sampled sections (e.g. 1:12)

In grafts containing high numbers of cells, an Olympus C.A.S.T grid system was used for stereology. The graft area was first defined before counting cells in a

random selection of regions within this area. As a 3D counting frame was not used, cell counts were adjusted with the Abercrombie correction. The total number of cells (C) in the graft per section were therefore calculated using the following formula:

Σc = total number of cells counted

ΣA = sum of all inclusion areas

Σa = sum of all sample areas

f = frequency of sectioning (e.g. 12)

M = section thickness

D = Average cell diameter

$C = \Sigma c \times (\Sigma A/\Sigma a) \times f/(D+M)$

For smaller surviving grafts, total cell counts for HuNu or NeuN were carried out using ImageJ. The phenotype of cells in transplants of mouse WGE in Chapter 6 was assessed with counts of DARPP-32. Total cell counts were estimated with the following formula, including the Abercrombie correction:

f*A*M/(D+M)

f = frequency of sectioning, A = cell counts for all sections, M = section thickness, D = Average cell diameter

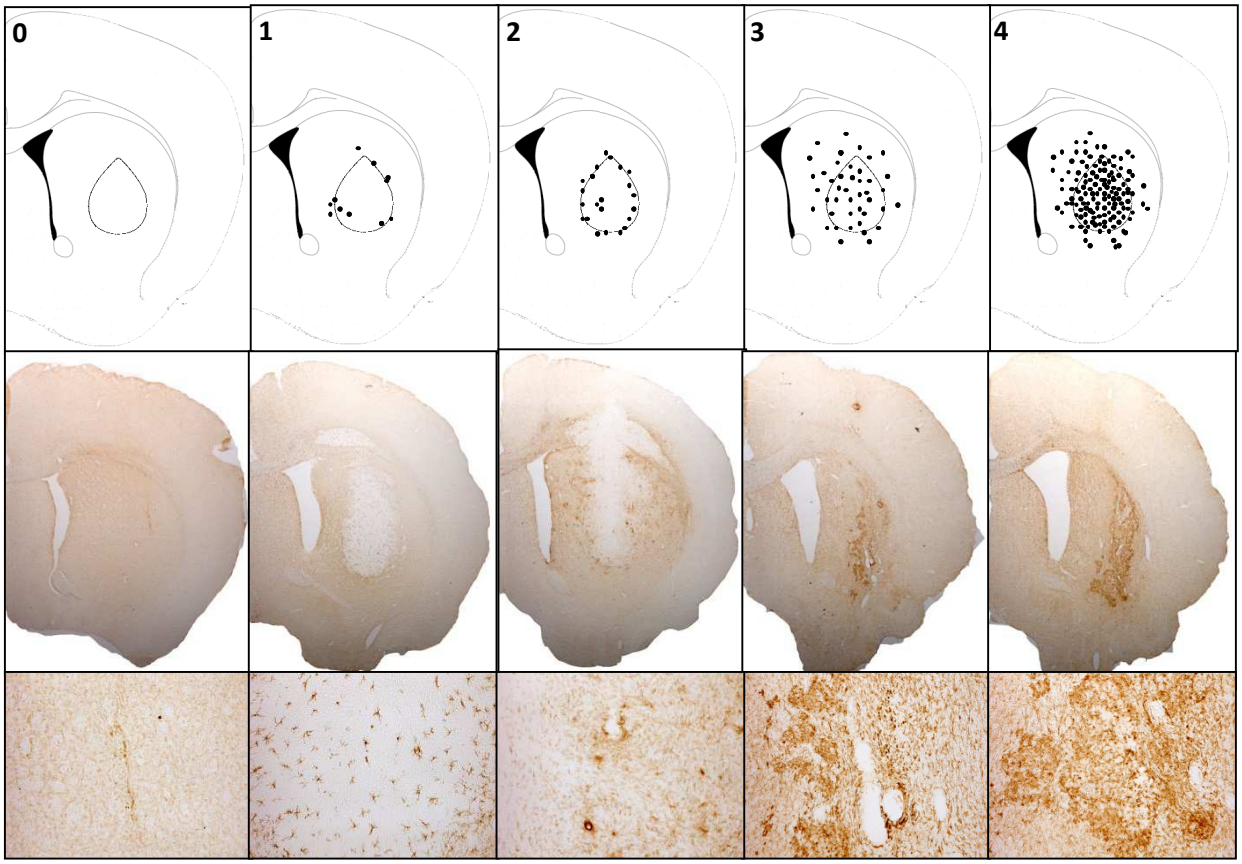
2.2.9 Semi-quantitative evaluation of host immune response to neural xenografts

To quantify the host T cell and microglial response to neural xenografts in all experiments, the extent of immunostaining for CD4, CD8 and CD11b (OX-42) was graded on a scale according to the defined criteria shown in **Figure 2.2** (Duan *et al.* 1995; Larsson *et al.* 1999)

2.2.10 Statistical analysis

All analyses were carried out using SPSS for windows statistical software (SPSS 20, *IBM*). The number of surviving grafts between experimental groups was initially compared using chi-squared analyses where possible. Quantification of

mean graft volume and mean total cell numbers within surviving grafts were compared by analyses of variance (ANOVA) as described in each chapter. Non-parametric data; semi-quantitative ratings of immune marker staining were compared with Kruskal-Wallis tests.



Grade	Criteria
0	No specific immunostaining in the graft area
1	Low number of positive cells, distributed as scattered single cells or clustered in a few small patches in or around the graft
2	Several positive cells distributed as single cells or clustered in multiple, prominent patches
3	Dense immunostaining of the graft area and a large number of positive cells in and around the graft
4	Very dense immunostaining of the whole graft area and a very large number of positive cells in and around the graft

Figure 2.2 Criteria for grading staining of immune cell markers (Duan *et al.* 1995)

Chapter 3

Comparing desensitisation using neural and non-neural tissue

3.1 Summary

The aim of the experiments included in this chapter was to investigate whether successful xenograft survival requires that neonatal desensitisation of host rats is performed using cells of the same type as those subsequently transplanted in adulthood. To date survival of human neural xenografts has been achieved using human neural cells to desensitise post-natally. Preliminary data suggested that using mismatched tissue types reduced xenograft survival. The ability to use non-matched cells, such as cells from another part of the foetus, adult cells, or renewable sources such as stem cells, would reserve scarce neural cells for grafting in adulthood. Furthermore, it would increase the likelihood of having a readily available source of cells for desensitising neonates, substantially facilitating transplant experiments and reducing animal wastage. Such experiments could also begin to clarify some of the mechanisms and limitations of desensitisation. The experiments reported here therefore assessed the survival of hPF cortical xenografts in the rat striatum in hosts desensitised neonatally with extra-neural cells compared to those in hosts desensitised with hPF cortical cells from the same donor embryo. Initial results suggested that successful desensitisation was more likely when using neural tissue for desensitisation. However further investigation comparing additional tissue types showed good survival of transplants, suggesting that it may not be necessary for the tissue used for desensitisation and transplantation to match, but that some tissue types, including liver and kidney, may be more effective for desensitisation of host rats to xenogeneic tissue.

3.2 Introduction

In the initial demonstration of successful neonatal desensitisation in rat hosts by Kelly *et al*, a number of different desensitisation protocols were tested (**Table 3.1**). These experiments suggested that the use of neonatally administered human liver cells to induce desensitisation to a subsequent transplant of hCTX tissue was less successful than that of neural tissue; 62% graft survival compared to up to 87%, although survival of transplants in hosts desensitised to neural tissue ranged between 66%-87% (**Table 3.1**; from Kelly *et al.* (2009b)). Routine studies carried out in rat hosts in this group have therefore used human neural tissue to desensitise neonatally and promote the survival of later transplants of neural tissue. Although the use of matched tissue has been essential to demonstrate that survival of transplanted cells can be achieved without additional immune suppression, it would be preferable to be able to use an alternative, less valuable source of tissue to desensitise host neonates. This would make available more tissue both for transplantation and desensitisation, particularly if neonatally injected cells could be derived from a renewable source of cells such as human FNPs. Additionally, it is possible that a wider spectrum of human cells, or "any" human cell type may be sufficient to induce desensitisation, which would open up possibilities even further.

Previous investigations of the phenomenon of neonatal tolerance have demonstrated successful tolerance induction to peripheral allografts in mice following neonatal injections of spleen, liver, and bone marrow cells (Adkins *et al.* 2004; Modigliani *et al.* 1997; West *et al.* 1994). Hosts in these studies were demonstrated to be tolerant to allogeneic donor cells by the survival of donor skin grafts or cardiac allografts and a lack of host cytotoxic T lymphocyte responses to donor spleen cells *in vitro* (Adkins *et al.* 2004; Modigliani *et al.* 1997; West *et al.* 1994). Although this has not been successfully replicated in a discordant xenograft paradigm, such as human to rat/mouse, it has been shown that under certain conditions tolerance can be induced to xenogeneic antigens (Borenstein *et al.* 2004). Tolerance was successfully induced in host mice in this study to donor cells from transgenic mice expressing a human MHC Class I allele (Borenstein *et al.* 2004). Various mechanisms have been proposed for the induction of neonatal tolerance, with Matzinger and colleagues highlighting the potential importance of the specific

population of cells injected to induce neonatal tolerance, suggesting that the types of cells used for inoculation would dictate whether an immunising or tolerising response ensued (Matzinger 1994; Ridge *et al.* 1996).

Tissue injected (i.p.) neonatally (P0-1)	**Tissue transplanted to adult striatum**	**Survival after transplant (weeks)**	**Number of grafts surviving (%)**
hPF cortex	hPF cortex	40	48/55 (87%)
hPF striatum	hPF striatum	25	10/15 (66%)
hFNP	hFNP	12	10/13 (77%)
hES-N	hES-N	12	9/12 (75%)
hFNP	hPF cortex	12	11/13 (85%)
hPF cortex	hFNP	12	11/14 (79%)
Human liver	hPF cortex	12	8/13 (62%)
hPF cortex	hPF striatum	10	10/12 (83%)

Table 3.1 Previously tested desensitisation protocols; adapted from Kelly *et al.* (2009b). Showing successful desensitisation using primary neural tissues, (hPF cortex and striatum), hFNP, hES and human liver in various combinations. hPF cortex injected neonatally shows the highest rate of survival with adult transplants of hPF cortex (87%). hPF = human primary foetal tissues hFNP = human foetal neural precursors derived from human primary foetal cortex, hES-N = embryonic stem cell derived neural precursors

To investigate whether desensitisation could be achieved with alternative hPF tissues, an initial experiment was carried out comparing the use of neural tissue and non-neural tissue (skin) from the same foetal donor to desensitise SD rats. Hosts received transplants in adulthood of hCTX tissue to the intact or QA lesioned striatum, and survival was assessed at a short time point (6 weeks) following transplantation. This was followed up in a subsequent experiment with the comparison of the survival of hCTX transplants to the intact striatum in rat hosts desensitised with three different donor tissue types from one human foetal donor; liver (hLiver), kidney (hKidney) and cortex (hCTX) 12 weeks after transplantation. Since graft survival was found to be comparable in transplants delivered to the intact and QA lesioned striatum, all transplants in this study were delivered to the intact striatum. Survival was assessed at 12 weeks in this case to demonstrate that grafts

were surviving due to effective desensitisation and that in previous studies a longer rejection response wouldn't have resulted in graft destruction past 6 weeks. Successful desensitisation with non-specific human donor tissue allows inferences to be made about the mechanisms of desensitisation, suggesting the antigen recognised is "human" but not related to tissue type.

3.3 Experimental Design

3.3.1 Desensitisation and transplantation of rat hosts

Table 3.2 outlines the design of the first experiment; detailed methods for all procedures are presented in **Chapter 2**. Experiments were carried out in two parts. Firstly; hosts were desensitised with hCTX (n=13) and human skin tissue (hSkin) (n=12) from the same foetal donor. Neonatal hosts were obtained from 2 pregnant SD rats (Harlan). In adulthood, at ages 8-12 weeks, half received QA lesions and all were transplanted with hCTX from a separate foetal donor. Survival was assessed at 6 weeks post transplantation (**Table 3.2**).

Neonatal injection of hCTX tissue ($1x10^5$ cells i.p.). (n=13)		Neonatal injection of hSkin tissue ($1x10^5$ cells i.p.) (n=12)		P2
↓		↓		8-12 weeks
QA lesion to right striatum (n=7)	No lesion (n=6)	QA lesion to right striatum (n=6)	No lesion (n=6)	
↓		↓		
Unilateral intrastriatal transplant of hCTX cell suspension ($5x10^5$ cells)				
↓		↓		6 weeks post transplant
Animals sacrificed and brains cut for histological examination				

Table 3.2 Experimental design; comparing graft survival at 6 weeks post transplantation in rat hosts desensitised neonatally with hCTX or hSkin cells

For the second experiment; hosts were desensitised with hCTX (n=11), hLiver (n=10) and hKidney (n=10) from one foetal donor followed by transplants to the intact adult striatum of hCTX from a different foetus, and survival was assessed 12 weeks after transplantation (**Table 3.3**). Neonatal hosts for this study were obtained from 5 pregnant SD rats (Harlan). No animals in either experiment received immunosuppression and cells were all delivered neonatally as i.p. injections in 1µl DMEM/F12.

Neonatal injection of hCTX tissue ($1x10^5$ cells i.p.). (n=11)	Neonatal injection of hLiver tissue ($1x10^5$ cells i.p.) (n=10)	Neonatal injection of hKidney tissue ($1x10^5$ cells i.p.) (n=10)	P1
↓	↓	↓	12 weeks
Unilateral intrastriatal transplant of hCTX cell suspension ($5x10^5$ cells)			
↓	↓	↓	12 weeks post transplantation
Animals sacrificed and brains cut for histological examination			

Table 3.3 Experimental design; comparing graft survival at 12 weeks post transplantation in rat hosts desensitised neonatally with hCTX, hLiver or hKidney cells

3.3.2 Histology and Immunohistochemistry

At 6 or 12 weeks post-transplantation (according to experimental design) animals were transcardially perfused and brains processed histologically as described in **Chapter 2**. A 1:12 series of sections were stained with CV and further 1:12 series were processed for single label immunohistochemistry using the primary antibodies listed in **Appendix 4.** A human specific antibody (HuNu, Millipore, UK) was used to identify surviving human cells and various immune markers were stained to assess the host response. The same basic protocol was used for all antibodies and is outlined in **Chapter 2**. Briefly, sections were quenched in 10% methanol and hydrogen peroxide before a 1 hour blocking step in 3% normal serum. Primary antibodies were incubated overnight with 1% normal serum followed by 2 hour incubation in secondary antibodies and 2 hour incubation in streptavidin ABC kit. Since all were mouse antibodies, biotinylated anti-mouse IgG (1:200, Vector) secondary antibody was used. Staining in all cases was visualised using the DAB method.

3.3.3 Quantification of graft survival and host immune response

Surviving grafts were identified initially with CV staining and confirmed with HuNu. Graft survival at both 6 and 12 weeks after transplantation was compared

with Chi-square tests. Counts of surviving transplanted human cells as identified by HuNu staining were carried out using stereological techniques for survival assessed after 6 weeks. Positively stained cells were counted in each section of a 1:12 series, allowing the number of surviving cells per graft to be calculated as described in **Chapter 2**. These could then be compared between animals tolerised with different tissue types. To assess survival of 12 week transplants from the second experiment, graft volumes were estimated by tracing the graft area as stained by HuNu on consecutive sections through the striatum on Image J. Total cell numbers and graft volumes were compared by one-way ANOVA. To quantify the host T cell and microglial response to neural xenografts in all experiments, the extent of immunostaining for CD11b (OX-42), CD8 and CD4 where possible, was graded on a scale (0-5) described in detail in **Chapter 2** (Duan *et al.* 1995; Larsson *et al.* 1999). Gradings of these markers were compared with non-parametric Kruskal-Wallis tests.

3.4 Results

3.4.1 Experiment 1: Graft survival at 6 weeks post transplantation in rat hosts tolerised with hCTX or hSkin

Surviving grafts were initially identified in Nissl-stained sections using CV and staining with HuNu confirmed the survival of transplanted human cells (**Figure 3.1**). At 6 weeks post transplantation, hCTX tissue transplanted into non-immunosuppressed adult rat brains was seen to survive in animals which had been desensitised neonatally with both hCTX and hSkin tissue. No significant difference was found in graft survival between hosts with QA lesions and those which received transplants to the intact striatum, χ^2 (1, N = 25) = 1.93, p = 0.165, therefore data was compiled to analyse the effect of desensitisation group on graft survival. Some healthy surviving grafts were found in both groups, although 62% of grafts survived in animals desensitised with hCTX tissue compared to 17% in animals desensitised with hSkin tissue (**Figure 3.2**). However, this difference was not found to be statistically significant; χ^2 (1, N = 25) = 3.532, p = 0.060, which is likely to be due to the small numbers of surviving transplants in this experiment, since a comparison of the data in **Figure 3.2B** shows a strong trend towards greater survival in hosts desensitised with hCTX. Mean surviving cell numbers and graft volume are displayed in **Figure 3.3** and **Figure 3.4**, showing larger surviving grafts in hosts desensitised with hCTX. Graft volumes and cell counts were not compared, since there were only two surviving grafts in hosts desensitised with hSkin.

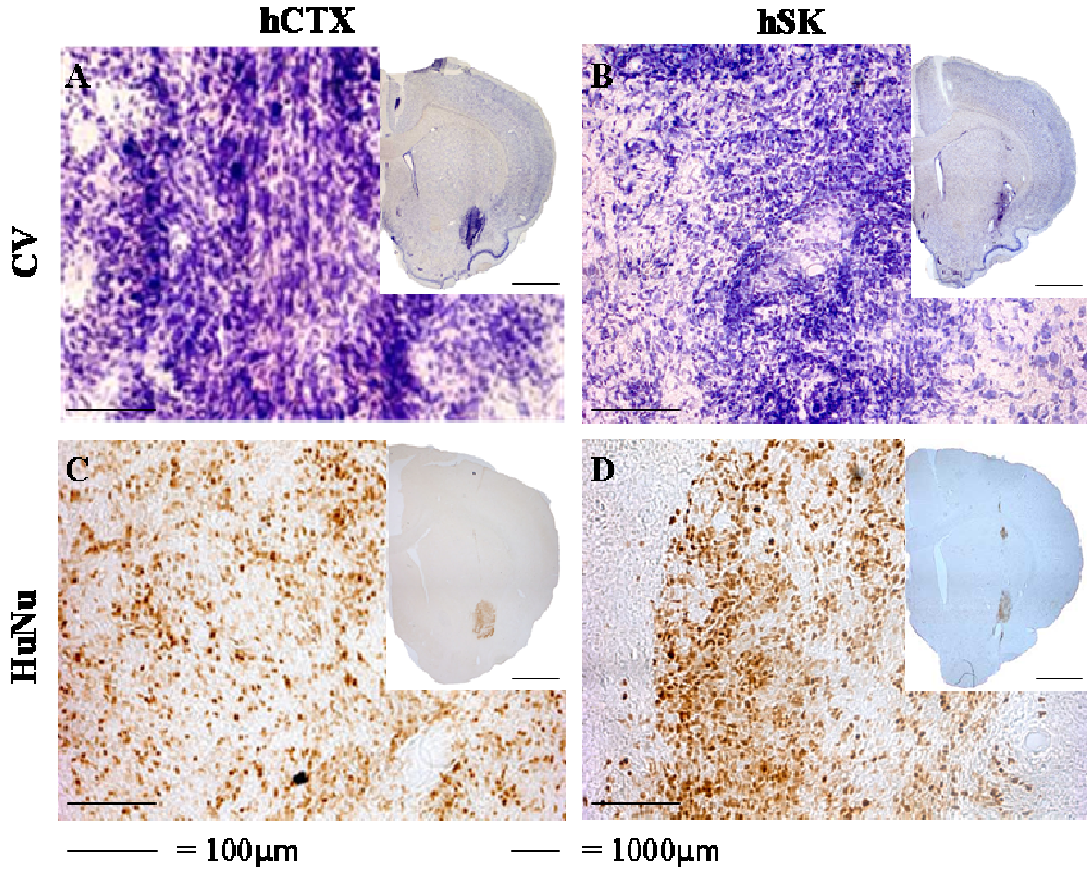


Figure 3.1 Photomicrographs of sections from rat hosts desensitised with neural (A, C) and non-neural (B, D) tissue stained with CV (A, B) and HuNu (C, D) and transplanted with hCTX into the intact striatum at 6 weeks post transplantation. Lower magnification images are inset showing small surviving transplants in the right striatum

A

Tissue injected neonatally	Lesion	Number of surviving grafts (%)	Total (%)	Mean cell number ($HuNu^+$)	Combined mean cell number	Mean graft volume (μm^3)	Combined mean graft volume (μm^3)
hCTX	QA	3/7 (42.8%)	61.5%	$25.1x10^4$	$20.9x10^4$	$11.7x10^8$	$9.6x10^8$
	None	5/6 (83.3%)		$16.7x10^4$		$8.3x10^8$	
hSkin	QA	0/6 (0%)	16.6%	-	$9.4x10^4$	-	$4.9x10^8$
	None	2/6 (33.3%)		$9.4x10^4$		$4.9x10^8$	

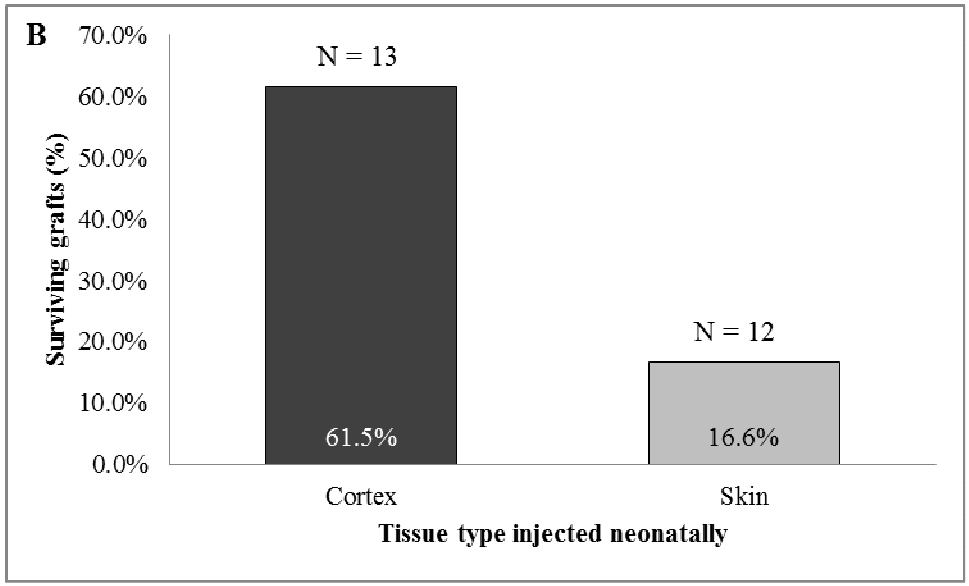


Figure 3.2 A) Graft survival at 6 weeks post transplantation.
B) Percentage of surviving hCTX transplants in both lesioned and unlesioned hosts 6 weeks after transplantation in adult rat hosts desensitised neonatally with neural or non-neural tissue. *Survival percentages are displayed at the base of bars, with total numbers shown at the top*

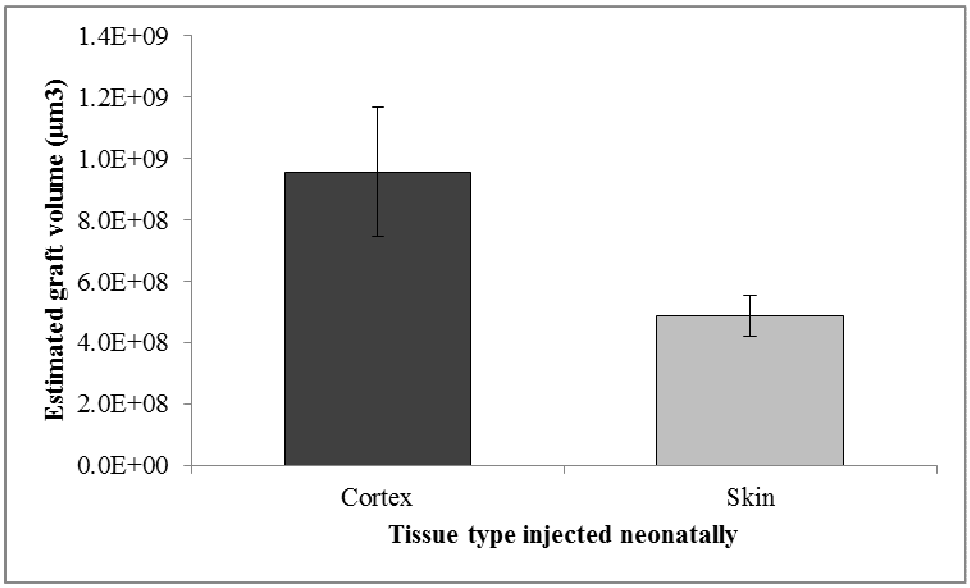


Figure 3.3 Mean graft volume as measured with HuNu+ staining 6 weeks after transplantation (excluding rejected grafts)

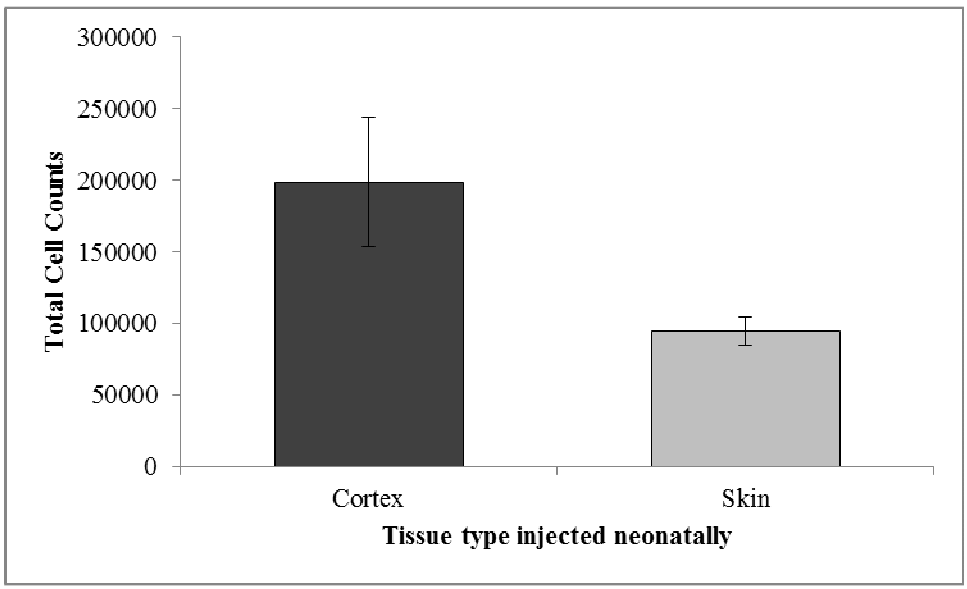


Figure 3.4 Mean numbers of surviving human cells 6 weeks after transplantation in adult rat hosts desensitised neonatally with neural or non-neural tissue (rejected grafts excluded)

3.4.2 Host immune response to neural xenografts 6 weeks after transplantation

Sections (1:12 series) at 6 and 12 weeks post transplantation were stained for rat T cell (CD4, CD8) and microglial markers (CD11b – OX42) in order to determine the extent of the immune response to human neural tissue grafts in animals which were desensitised neonatally with neural and non-neural human embryonic tissue. **Figure 3.5** shows staining with CD11b (OX42, Serotec) specific for activated rat microglia/macrophages, and $CD8^+$ T cells. In these examples, staining can be seen along the needle tract and surrounding the grafted area. CD11b staining was graded and differences compared with a non-parametric Kruskall-Wallis test, showing no difference in the host microglial response to surviving xenografts in hosts desensitised with neural vs non-neural tissue; H (1) = 0.21, p = 0.885 (**Table 3.4**). Staining for CD4 is not shown, and grading was not carried out for either T cell marker, as due to issues with storage of tissue sections described in **Appendix 5**, not all sections could be stained successfully and quantified.

Desensitisation/Grade	**0**	**1**	**2**	**3**	**4**
hCTX		+	++++	++++++++	+
hSkin		++++++++	+	+++	

Table 3.4 CD11b immunoreactivity in sections from animals desensitised with hCTX or hSkin cells and transplanted with hCTX at 6 weeks post-transplantation. Each + corresponds to one host; a red + denotes a rejected graft.

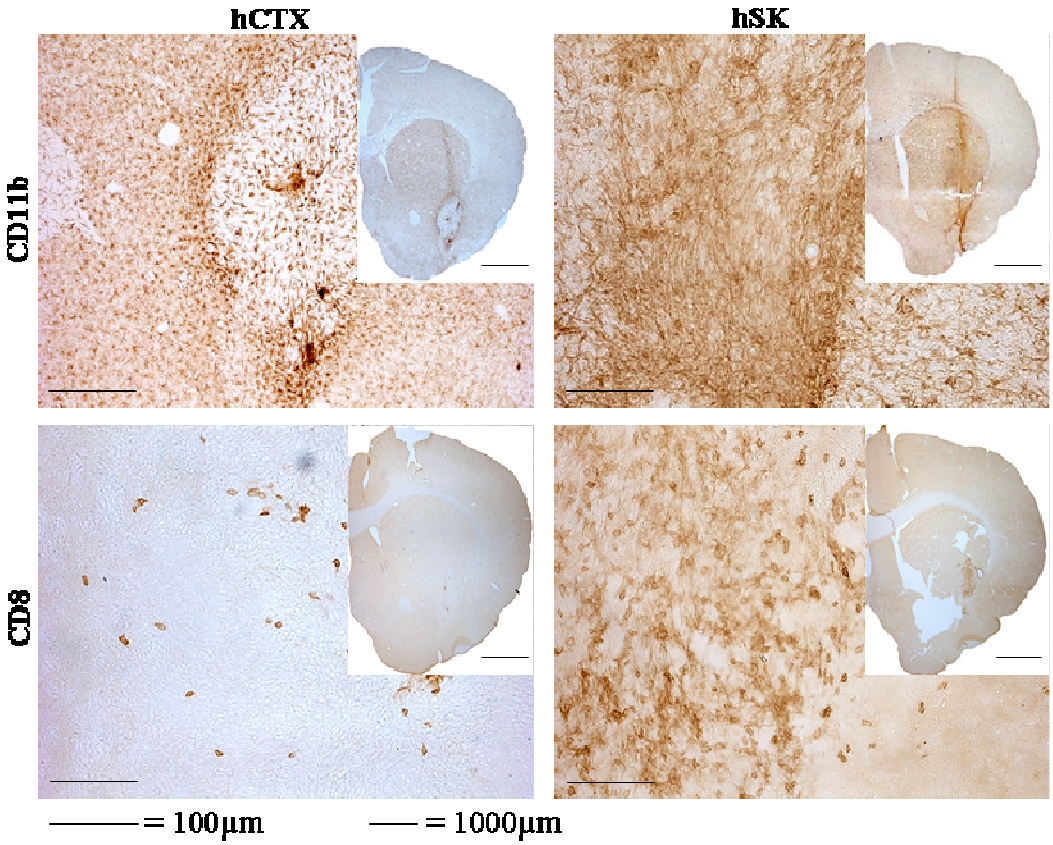


Figure 3.5 Tissue sections from hosts desensitised with hCTX or hSkin, tissue and transplanted with hCTX stained for $CD8^+$ T cells and $CD11b^+$ microglia at 6 weeks post transplantation. Staining shows infiltration of microglia and T cells in the grafted area. These examples show more severe infiltration in the host desensitised with hSkin, however there were no differences in immune response between the two groups.

3.4.3 Experiment 2: Graft survival at 12 weeks in rat hosts transplanted with hCTX, hLiver and hKidney

Successful graft survival in hosts desensitised with non-neural tissue was demonstrated in this experiment as identified with CV and HuNu staining, with 100% of transplants surviving in both non-neural groups (liver and kidney). However, only 36.4% of transplants in those desensitised with human cortical tissue were found to survive (**Table 3.5** and **Figure 3.8**). Surviving grafts in the majority of hosts were found to be very large, spanning the whole striatum (**Figure 3.9**). A chi-squared test showed there to be significantly more grafts surviving in hosts desensitised with liver or kidney cells than those desensitised with hCTX; χ^2 (2, N = 31) = 16.44, $p < 0.001$. Graft volume was calculated from areas measured using Image J (**Figure 3.6**). No differences in graft volume were found between hosts desensitised with different donor tissue types (F (2,21) = 0.347, p = 0.711).

Tissue injected neonatally	Number of surviving grafts	Total (%)	Mean graft volume (µm^3)
Liver	10/10	100%	5.2x10^7
Kidney	10/10	100%	5.4x10^7
Cortex	4/11	36.4%	3.6x10^7

Table 3.5 Surviving human transplants in desensitised hosts 12 weeks after transplantation

Figure 3.6 Estimated mean graft volumes of surviving transplants in desensitised hosts 12 weeks after transplantation

3.4.1 Host immune response to neural xenografts 12 weeks after transplantation

To investigate the host immune response to transplants of hCTX, staining was carried out for T cell (CD8+ and CD4+) and microglial (CD11b) markers (**Figure 3.8**) and quantified according to the grading system described previously (**Table 3.6**). This is represented graphically in **Figure 3.7**. Non-parametric Kruskall-Wallis tests were carried out for all immune marker grading of sections containing surviving grafts. No significant differences were found between desensitisation groups for any stains; test statistics are shown in **Table 3.6**.

Stain	Group	Grade					*H*(2)	*p*
		0	**1**	**2**	**3**	**4**		
	hLiver	++	++++	+	+++			
CD8	**hKidney**	+++	+++++		+	+	0.957	0.620
	hCTX	+++++	+++++			+		
	hLiver	++	++	+++	+++			
CD4	**hKidney**	+	+++++	+++		+	1.327	0.515
	hCTX	++	++++	+++		+		
	hLiver		++++	++++++				
CD11b	**hKidney**		+++++++	++	+		1.150	0.563
	hCTX	+++++	+++		+			

Table 3.6 Grading of immune marker staining in sections from rat hosts desensitised with different human tissue types and transplanted with hCTX at 12 weeks post-transplantation. A + represents the highest score for each rat with red + indicating hosts with rejected grafts.

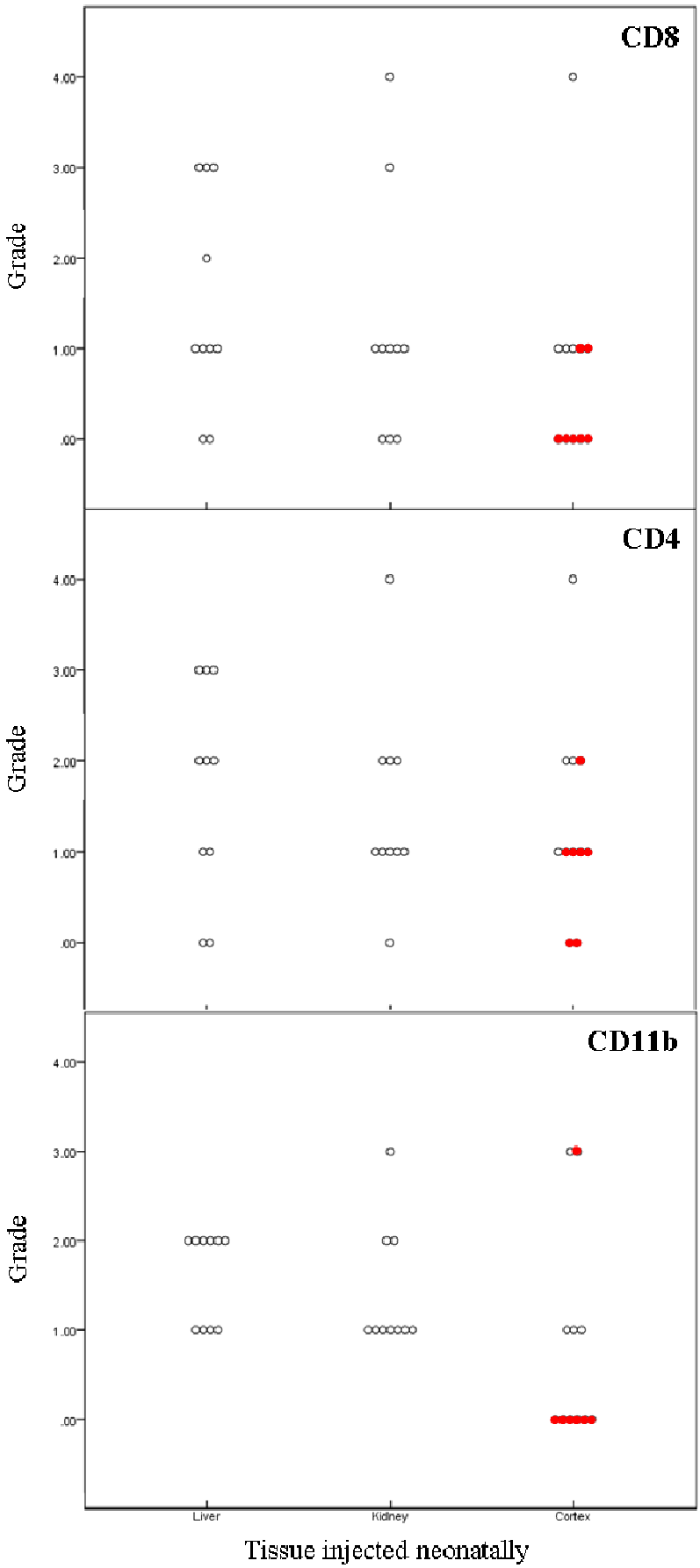


Figure 3.7 Grading of immune marker staining in rat hosts desensitised with different tissue types. Each circle indicates the highest graded section for each animal. Red circles denote sections without surviving grafts.

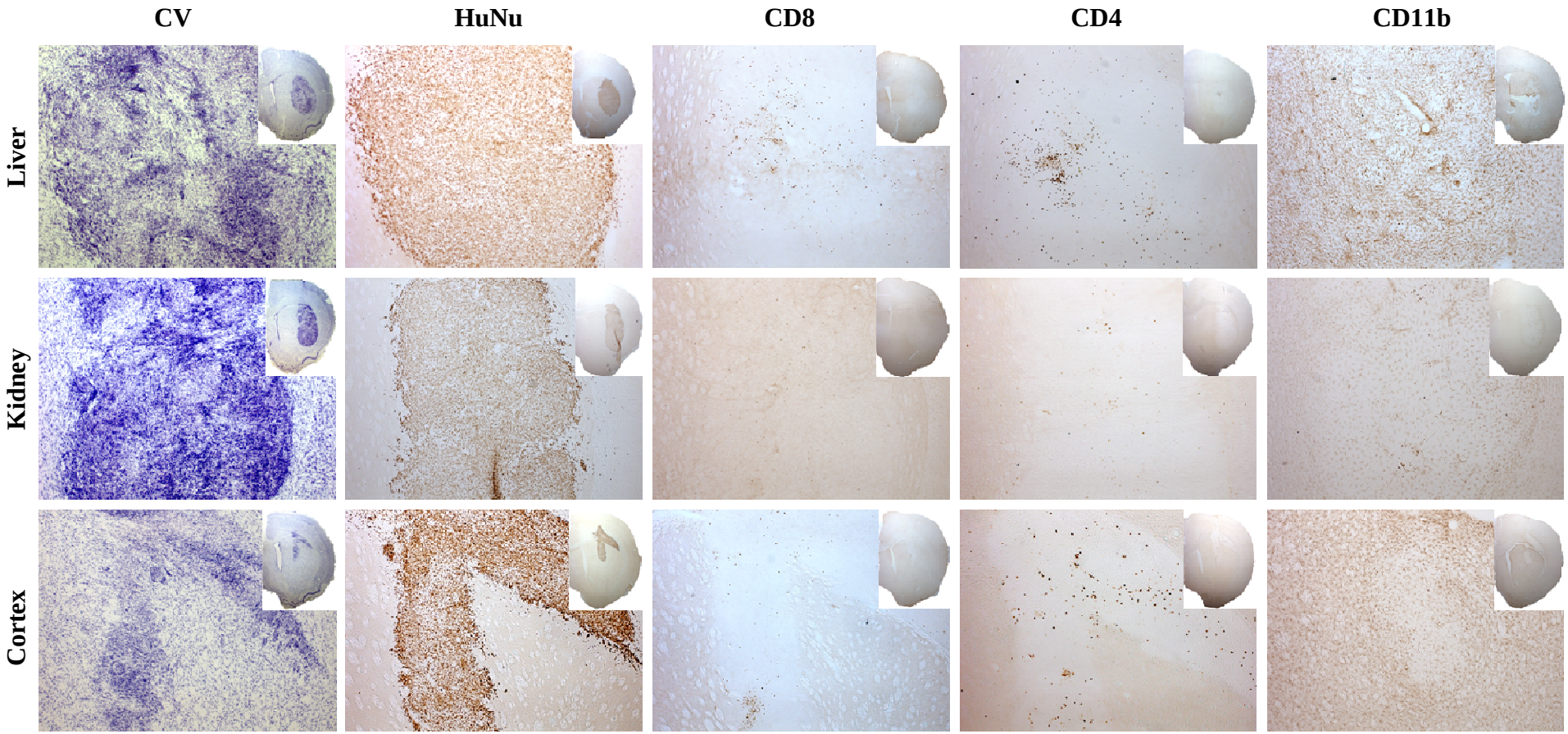


Figure 3.8 Photomicrographs of sections from rat hosts desensitised with human liver, kidney, and cortex showing survival of transplants and the host immune response 12 weeks after transplantation. Images show large surviving grafts in desensitised hosts, with some labelling of T cells and minor infiltration with microglia.

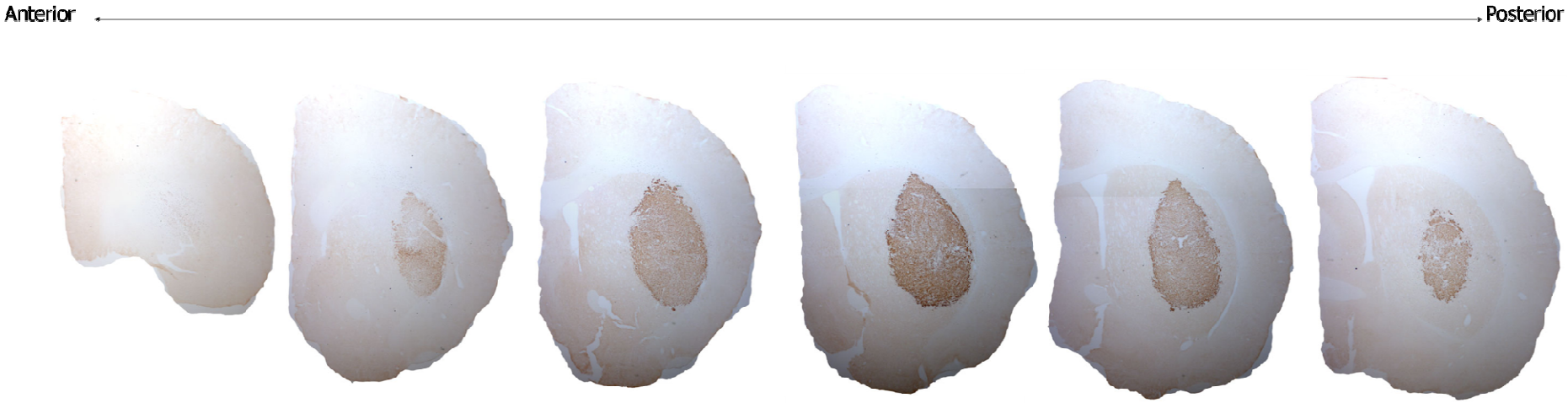


Figure 3.9 A representative surviving hCTX graft in a host desensitised neonatally with hLiver, showing that surviving hCTX transplants were large, filling the whole striatum

3.5 Discussion

The aims of the experiments described in this chapter were to determine whether it is necessary to use the same tissue type to desensitise hosts neonatally as is used to transplant in adulthood, or whether it is sufficient to use other tissues from the same donor species. Being able to desensitise with alternative tissue would reserve scarce neural tissue needed for transplantation as well as giving more insight into the mechanisms of the method and specificity of the desensitisation. In Experiment 1, host rats were desensitised with differing tissue types (hCTX or hSkin) at birth, but all transplanted with the same tissue (hCTX) in adulthood in order to compare survival between the two groups. The findings from this study suggest that better survival may be achieved with transplants of matched tissue, indicating that the tissue used for desensitisation should be of the same type as that used to graft. A second experiment investigated the potential of other hPF tissue types for use in the desensitisation of rat hosts. Neonatal rats were desensitised with hCTX, hLiver, or hKidney, and again all received transplants of hCTX in adulthood. This study showed relatively poor survival of transplants in hosts desensitised with hCTX as compared to 100% of grafts surviving in the other two groups, however this was still only 25% less survival between Experiment 1 and 2.

The findings from both experiments therefore do not suggest that the tissue used for desensitisation must be the same as that used to transplant, rather that the results may be variable dependent on the type of tissue used. It is not clear why survival in the second experiment of this chapter was so poor in hosts desensitised with hCTX, although this may be an anomaly due to poor quality cortical tissue from this specific donor foetus since previous experiments, including some presented in this thesis, have shown more consistent survival to a degree which may be achieved with conventional immune suppression (Kelly *et al.* 2009b). The previous finding that liver was less successful as a donor source for desensitisation may also be due to this variability. Additionally, the previous finding that 62% of grafts survived following desensitisation with liver cells still shows that survival is better than may be expected with no treatment and not far from the 75% of grafts which survived following daily treatment with CsA (Kelly *et al.* 2009b). The variable survival of

different tissue types in these experiments may be explained to an extent by considering previous work in the field of neonatal tolerance.

Neonatal (and in utero) tolerance induction has been described for decades using a similar method to that described here (Billingham and Brent 1956; Billingham *et al.* 1953). Previous work shows the induction of tolerance to allogeneic skin grafts following an injection of spleen (Adkins *et al.* 2004; Ridge *et al.* 1996), liver (West *et al.* 1994) or bone marrow cells (Modigliani *et al.* 1997) though less success has been observed in the case of xenografts (Shen *et al.* 1996). This may be due to species differences in T cell receptor recognition of xenogeneic MHC molecules; although this suggestion has been rejected by Borenstein et al (Borenstein *et al.* 2004) who found that tolerance to xenogeneic MHC molecules could be induced in B6 mice, promoting survival of skin grafts. Donor spleen cells ($1.5x10^7$) from a transgenic mouse strain expressing the human MHC class I allele human leukocyte antigen (HLA)-B7 were injected intravenously (i.v) to induce tolerance in normal B6 mice, therefore demonstrating that the host immune system was capable of recognising donor xenogeneic MHC molecules (Borenstein *et al.* 2004).

Some mechanisms for the induction of neonatal tolerance to allogeneic tissue have been proposed, although a definitive conclusion has not been reached. Passive models suggest that, as with natural self-tolerance, neonatal tolerance occurs through negative selection of self-reactive T cells. The presentation of donor antigens in the thymus, along with self antigens therefore allows donor reactive thymocytes to also be negatively selected. As few mature T cells exist neonatally, donor cells are able to reach the thymus to promote this deletion of donor reactive T cells (Morrissey *et al.* 1983; Ridge *et al.* 1996). Active models suggest that newly developing T cells present in the neonatal host generate mainly TH_2 responses, protecting from self recognition and also reducing recognition of donor cells present at this stage (Bandeira *et al.* 1989).

However, Matzinger and colleagues highlight the ability of neonates to become immunised rather than tolerised to neonatally presented viruses (Miller *et al.* 1994) which does not agree with these models (Ridge *et al.* 1996). They therefore propose a third model, the “Danger” model suggesting that it is not the host population of T cells which governs the induction of tolerance, rather it is what constitutes the

population of injected donor cells which determines tolerance or immunity (Matzinger 1994; Ridge *et al.* 1996). When the population of cells used for neonatal administration does not include a large proportion of mature APCs, a lack of co-stimulation of host T cells occurs resulting in a tolerising response. Conversely, if a large population of active APCs such as DCs are present in the injected suspension, an immunising effect will occur. The authors found exactly these responses when injecting spleen cells or isolated DCs (Ridge *et al.* 1996). Tolerance induction was found when injecting a large number of spleen cells neonatally, whereas injecting an enriched population of DCs primed T lymphocytes for attacking donor cells. Much variation has been found in this area, with some disagreement for this proposal from a study showing that neither donor T nor B cells are required for the induction of neonatal tolerance, since successful tolerance has been induced from injection of spleen and bone marrow cell suspensions from *Rag1*$^{-/-}$ mice which contain neither of these populations, but may contain professional APCs (Chan *et al.* 2007; Modigliani *et al.* 1997).

In the context of the experiments described in this chapter, a suspension made up of cells from neural tissue, including hCTX, is unlikely to include a large population of mature APCs, since DCs only migrate to the CNS or mature from resting microglia as a response to pro-inflammatory signals (Santambrogio *et al.* 2001; Shrikant and Benveniste 1996). Additionally hCTX taken at this stage in embryonic development is likely to be a highly proliferative source of tissue, as confirmed by the large surviving transplants and positive staining for Ki67 found in transplants as documented in **Chapter 4** (See **Appendix 6**). The presence of a viable source of proliferating cells which are not rejected by the immature neonatal immune system will potentially provide a constant source of antigen to be processed as self during immune system development. It is possible that the non-neural tissue suspension in the first experiment of this chapter, made up of donor skin cells, contains a higher number of resident DCs (Langerhans cells) which are present in the skin, increasing the chance of an immunising effect. This more closely agrees with the suggestion that the presence of donor APCs may be more likely to have an immunising rather than a tolerising effect. It also suggests that other types of tissue, which does not necessarily match that used to transplant in adulthood, could still be

used to successfully desensitise host animals to xenogeneic tissue, as in the case of neonatal tolerance to allogeneic tissue in mouse hosts.

The second experiment described in this chapter aimed to determine whether this was the case by investigating the survival of hCTX transplants in hosts desensitised using hLiver and hKidney cells. These tissues were chosen in part as an 'alternative' tissue source for injection as opposed to cortex or skin cells. Liver was more specifically chosen due to its successful use in the induction of neonatal tolerance to allografts in mice (West *et al.* 1994). Whilst, spleen would have been an ideal source of donor cells based on findings from previous neonatal tolerance research (Adkins *et al.* 2004; Ridge *et al.* 1996), this proved difficult to obtain from the human embryos at the ages available. Successful desensitisation with liver cells in this experiment, with 100% survival of hCTX grafts is congruent with these findings, despite the use of donor cells from different embryos. It has previously been noted that liver transplants are more readily accepted in a number of mammalian species including pigs and rats, even across MHC differences, and can even have a tolerising effect in adult hosts (Calne 2000; Calne *et al.* 1969; Calne *et al.* 1967). It has been suggested that this is due to the nature of the APCs present in the liver, in particular in the human liver. Kupffer cells, the liver's macrophage population, myeloid DCs, and liver sinusoidal endothelial cells (LSECs), have all been shown to secrete immunosuppressive tolerance inducing signals including IL-10; which down-regulate MHC-II, CD80 and CD86 (Knoll *et al.* 1995; Sato *et al.* 1996). Additionally, induction of Tregs has been identified by liver APCs, which may also contribute to immune tolerance (Moseman *et al.* 2004). These factors combined with the nature of the immature neonatal immune system may be conducive to the induction of tolerance in our host rats. For a review on these findings, see Crispe (2011). Although suggestive of reasons for why desensitisation with liver cells may have promoted survival of hCTX transplants, the same findings have not been observed with kidney, thus the reason for this finding is unclear.

3.6 Conclusions and future work

Findings from these experiments demonstrate that it is possible to use alternative, non-neural hPF tissue types to desensitise hosts to human tissue to a sufficient degree for promotion of survival of hCTX transplants. The data also suggest that using other types of tissue may provide more consistent survival of hCTX grafts to the rat striatum up to 12 weeks after transplantation. Replication of these findings is required to confirm whether this is the case, as well as confirmation with human WGE transplants to show that desensitisation with these tissue types is an appropriate method to promote survival of striatal tissue in a rat HD model, and that survival of hCTX tissue is not more likely due to the rapid proliferation of these cells. Further experiments using alternative tissue such as NPCs expanded in culture for desensitisation would also be valuable. This could provide a more constant source of cells with which to desensitise neonatal host animals, avoiding difficulties with the acquisition of hPF tissue.

Transplantation studies can show whether desensitisation with these alternative tissue types is sufficient to promote long term survival of hCTX transplants to the rat striatum and allow inferences to be made regarding the potential mechanisms. However in order to confirm whether the population of cells itself influences the efficacy of desensitisation it is necessary to characterise the donor tissue. Therefore, future work aims to analyse the differing donor tissue types with fluorescence activated cell sorting (FACS) analysis to determine the presence of mature APCs as measured by expression of MHC-II and CD11c (Shortman and Liu 2002; Shrikant and Benveniste 1996). Additionally it would be valuable to determine whether desensitisation is induced as a result of the presence of cells of a hematopoietic lineage inducing chimerism in hosts, since this has frequently been suggested as a requirement for, or at least to strongly correlate with successful neonatal tolerance in mice (Borenstein *et al.* 2004; Chan *et al.* 2007). This could be achieved by assessing the presence of human cells in host lymphoid organs. Collecting data from these studies along with data from transplant survival in hosts desensitised with these populations of donor cells will provide more understanding of which population of cells administered neonatally induces the best cell survival and the potential mechanisms involved.

Another important question to be investigated is to the fate of the donor cells injected into neonatal hosts. This would give insights into the mechanisms involved, allowing determination of how long donor cells remain present in the host and how they are taken up by the neonatal rat immune system, as well as whether they ultimately induce some form of chimerism in the host. A number of possible methods may be used for such investigations. Potentially labelled cells could be combined with imaging techniques to track the location of injected cells. Or alternatively, as donor cells are of a different species origin, it may be possible to use antibody staining in histological sections to identify human cells within neonatal rat tissues. The main issue is initial determination of the location of cells, as there is no clear idea of distribution and hence no indication of where to begin looking within the neonate. An initial experiment has been designed to elucidate this matter, by collection of a number of organ samples at various time-points following injection and using reverse transcriptase-polymerase chain reaction (RT-PCR) to identify the presence of human genes within the rat host. Although this experiment has been carried out, due to difficulties with optimisation of PCR primers and protocols, data collection is not complete and the results cannot yet be analysed.

Ultimately, neonatal desensitisation will be required to promote the survival of transplants of human donor cells for sufficient time to complete full functional assessment of transplants. These experiments will aim to test donor cells derived from hESCs and hiPSCs, most likely in comparison to hPF tissue, demanding the demonstration of successful desensitisation to these cell types. Previous work has shown successful desensitisation (75%) up to 12 weeks in animals desensitised and transplanted with hESC-derived neuronally directed cells (Kelly *et al.* 2009b). Future work will determine whether desensitisation of rat hosts using hPF tissue can be used to promote survival of hESC derived neuronal cells directed towards a striatal phenotype for sufficient time to assess the potential of these donor cells (up to 40 weeks).

Chapter 4

Tolerance Specificity

4.1 Summary

To further comprehend the practical limitations and potential underlying mechanisms of neonatal desensitisation, the experiments in this chapter were designed to explore the specificity of the induced desensitisation in terms of the cells used to desensitise the animals and cells transplanted subsequently in adulthood. The objective was to investigate whether neural transplants of hPF tissue would only be tolerated following prior desensitisation to tissue of the same species. Demonstrating "tolerance" to be species specific would suggest that desensitisation is not a result of a general reduction in the sensitivity of the immune system, and that the host is still capable of rejecting a transplant from a different donor species. Survival of transplants of PF hCTX or mWGE, or both (bilateral- mWGE on one side and hCTX on the contralateral side) was compared in rat hosts desensitised with tissue from either the same or different species, or treated daily with CsA. Results were unexpected, in that no surviving mouse transplants could be identified in any condition but surviving transplants of hCTX were found in all groups, including hosts that had been desensitised with mouse tissue. hCTX transplant survival persisted where a contralateral transplant of mWGE was rejected in all hosts desensitised with hCTX or treated with CsA as well as some hosts desensitised with mWGE[*]. Data is suggestive of at least 50% survival of hCTX transplants in hosts desensitised with tissue from a different donor species, implying desensitisation to a common epitope present on mouse and human cells, or potentially a general reduction in immune sensitivity. Further work is required to identify the reason behind these findings, and these objectives are discussed in this chapter.

[*] Some tissue processing and histology for this experiment was carried out by a technician in the BRG; Tom Steward. All surgical techniques, and quantification and analyses of histological stains were carried out by myself

4.2 Introduction

A previous study by Kelly *et al* showed that rat hosts can be neonatally desensitised to human neural tissue, allowing long term survival of a subsequent transplant to the striatum in adulthood (Kelly *et al.* 2009a). This was demonstrated initially with transplants into the intact striatum of SD rats using a range of neural donor cells (regions of the primary foetal brain, FNPs, ESCs), but in most cases with the same types of cell being used for neonatal injection and striatal implantation in adult brain. However, Kelly *et al* also presented preliminary evidence that graft survival (hCTX) could be achieved when the neonatal injections were performed with cells from a different region of the foetus (liver). Further evidence that neural cells (primary foetal cortex) can survive following desensitisation to extra-neural tissue (liver, kidney and skin) is presented in **Chapter 3**. Taken together these findings provide further support for neonatal desensitisation as a model for pre-clinical testing of potential human donor cell types, but they also suggest a broader desensitisation than first assumed, with hCTX transplants surviving following desensitisation with a range of tissue types. This chapter seeks to explore this further, by identifying whether this desensitisation extends to tissue from different donor species, or whether it is specific to cells from the donor species which were used for neonatal inoculation.

This experiment was conducted in order to demonstrate that rats inoculated neonatally with hPF tissue have been specifically desensitised to human tissue, as opposed to survival of neural transplants being due to a global reduction in the host's capacity to mount an immune response to a neural xenograft. To achieve this, rat hosts were desensitised with either mWGE or hCTX. The pups were allowed to mature to adulthood and were then either transplanted unilaterally with cells from the same donor species as used for desensitisation or with a mismatching donor cell type, or were transplanted bilaterally with a graft of each tissue type. Since mice could be time-mated to produce a ready supply of foetal donor tissue, it was possible to use mWGE for desensitisation and transplantation. However, difficulties in the supply of hPF tissue meant that cortical tissue, which is much more plentiful than WGE, had to be used for both stages of the experiment in order to generate sufficient donor cells. Immune suppressed controls were included for all transplant groups to

confirm that any rejection seen was due to the mismatch of desensitisation/transplantation tissue and not due to problems with the transplanted tissue itself. It was hypothesised that unilateral transplants would survive following desensitisation to tissue from the same donor species, and that unmatched transplants would be rejected. To confirm that this was specific to desensitisation within one host animal, bilateral transplants were included, with the hypothesis that the matched species transplant would persist, whereas the unmatched transplant would be selectively rejected.

The interpretation of this study was complicated by the lack of survival of any mouse transplant in any condition. This made it impossible to assess the differential effect of species-matched and mis-matched conditions on the survival of mouse grafts. However, there was clear and unambiguous survival of human grafts, so this arm of the experiment could be analysed and revealed that desensitisation did not appear to be specific to the donor species tissue injected neonatally. Specifically, surviving hCTX transplants were found in hosts desensitised with mWGE. This survival was robust enough to persist, even in the presence of a contralateral rejected mWGE transplant, although fewer hCTX grafts survived following desensitisation to mouse cells than to human cells.

4.3 Experimental Design

SD rat hosts were desensitised (P0-2) to either mWGE or hCTX and transplanted with various combinations of donor tissues (see **Table 4.1**). For desensitisation; hCTX tissue was collected and dissected from one donor embryo, and mWGE was dissected from two time mated CD-1 mice at E14 and E15 (Harlan, UK). Desensitised hosts consisted of 34 neonates (18 male and 16 female; from 3 pregnant SD rats, Harlan). A group of hosts were not desensitised, and received the same transplant combinations with daily injections of CsA (10mg/kg) starting from one day prior to transplantation. These hosts arrived at >200g from the supplier (Harlan, n = 24). As desensitisation was dependent on the birth of rat pups at the same time as the presence of donor tissue, mWGE desensitised hosts were derived from two different litters which received neonatal injections from different litters of mouse embryos. Hosts were then randomly assigned to each transplant condition. All neonatal injections consisted of $1x10^5$ donor cells in 1 µl DMEM/F12.

Transplants were carried out in hosts >200g; in desensitised hosts this was between 8 and 16 weeks of age. Due to availability of human tissue, and time constraints, transplants had to be carried out on two separate occasions. CsA treated control groups were therefore divided between transplant groups to ensure differences were due to host treatment rather than donor transplant tissue. Donor tissue for hCTX transplants was collected and dissected from two embryos; CRL 35.5mm (group 1) and 46.7mm (group 2). Donor tissue for mWGE transplants was again collected and dissected from time-mated CD-1 mice at E14. All transplants consisted of $5x10^5$ donor cells in 1 µl DMEM/F12. Transplant survival in all cases was assessed 12 weeks after transplantation. To clarify the complex design of these experimental groups, the design is outlined in **Table 4.1**.

	Transplant group 1			**Transplant group 2**					
P0-2	**Desensitised mWGE**			**CsA**			**Desensitised hCTX**		
	⬇			⬇			⬇		
8-16 weeks	Transplant hCTX (n=6)	Transplant mWGE (n=6)	Transplant Bilat (mWGE/hCTX) (n=6)	Transplant hCTX (n=8: 4 per transplant group)	Transplant mWGE (n=8: 4 per transplant group)	Transplant Bilat (mWGE/hCTX) (n=8: 4 per transplant group)	Transplant hCTX (n=5)	Transplant mWGE (n=5)	Transplant Bilat (mWGE/hCTX) (n=6)
	⬇			⬇			⬇		
Graft survival assessed at 12 week post-transplantation									

Table 4.1 Design of experiment to determine whether desensitisation is specific to tissue from the donor species used for neonatal injection. All transplants were delivered to the intact striatum. The side of transplant delivery was counterbalanced across groups. hCTX = human primary foetal cortex, mWGE = mouse whole ganglionic eminence. Bilateral transplants consisted of an hCTX transplant to one striatum and an mWGE transplant to the other, again the side of transplant delivery was counterbalanced. All animals were desensitised with an i.p. injection of $1x10^5$ cells and transplanted with $5x10^5$ cells

4.4 Results

4.4.1 Graft survival 12 weeks after transplantation

CV staining was carried out to identify surviving grafts. The presence of surviving human xenografts could be confirmed with HuNu staining, however no definitive marker could be used to detect surviving mouse transplants. Despite this it was clear from CV staining that no mouse transplants survived in any treatment group, including CsA treated control animals (**Figure 4.1**). The percentage of surviving transplants in each group is represented in **Figure 4.1** and **Table 4.2**. As no transplants of mouse donor tissue survived, analysis was carried out only on surviving unilateral or bilateral human transplants from all treatment groups. Significantly more hCTX transplants were found to survive in hosts desensitised with hCTX than those desensitised with mWGE; χ^2 (5, N=39) = 13.65, $p < 0.05$ (**Table 4.2**).

The volume of surviving human grafts was measured from HuNu staining and is also shown in **Table 4.2**. In order to reduce heterogeneity of variance, log transformation was carried out on the data prior to analysis. Graft volumes and the number of surviving transplants are displayed in **Table 4.2**. Representative surviving hCTX transplants in CsA treated hosts from each transplant group are shown in **Figure 4.2.** The majority of surviving transplants were very large, with CV staining in swirls throughout the striatum. The centres of the grafts were found to contain proliferating cells as stained by Ki67 (See **Appendix 6**). A two-way ANOVA was conducted on the transformed data to determine the effect of transplant group and treatment on surviving hCTX graft volume. This revealed a highly significant main effect of transplant group; F (1, 25) = 15.970, $p < 0.001$ with larger graft volume seen in transplants from group 1, which received the earlier gestation tissue (**Figure 4.4**). No significant main effect of treatment was found; F (5, 25) = 0.286, $p = 0.916$. Additionally no interaction was found between transplant group and treatment; F (1, 25) = 0.016, $p = 0.901$, indicating that greater graft volumes observed in hosts desensitised with mWGE (**Figure 4.3**) were due to the donor tissue used in the different transplant groups rather than the effect of the treatment (desensitisation with hCTX/mWGE or daily CsA). **Figure 4.5** shows examples of rat host brain

showing the region where an mWGE graft has been lost, and typical staining seen in a bilateral graft.

Treatment group	Tissue Transplanted	Number of surviving human grafts (%)	Number of surviving mouse grafts (%)	Mean graft volume (μm^3)
DhCTX (neonatally)	hCTX	5/5 (100%)	-	$30.5x10^8$
	mWGE	-	0/6 (0%)	-
	Both (bilat)	6/6 (100%)	0/6 (0%)	$34.2x10^8$
DmWGE (neonatally)	hCTX	3/6 (50%)	-	$128.5x10^8$
	mWGE	-	0/6 (0%)	-
	Both (bilat)	4/6 (66.7%)	0/6 (0%)	$99.8x10^8$
CsA (from transplantation)	hCTX	8/8 (100%)	-	$74x10^8$
	mWGE	-	0/8 (0%)	-
	Both (bilat)	8/8 (100%)	0/8 (0%)	$79.2x10^8$

Table 4.2 Percentage of surviving transplants in hosts desensitised and transplanted with hCTX or mWGE, and the mean graft volume of hCTX grafts. DhCTX = Desensitised neonatally with hCTX tissue; DmWGE = Desensitised neonatally with mWGE tissue; CsA = treated daily with CsA.

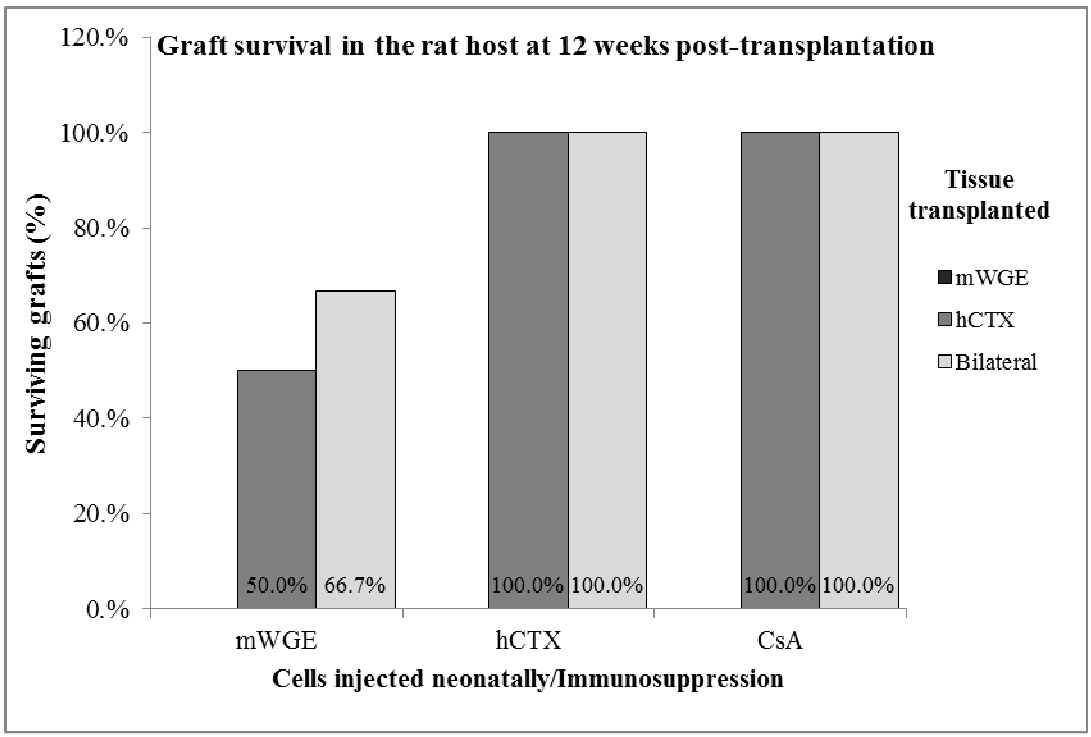


Figure 4.1 Survival of hCTX grafts in hosts desensitised with mWGE or hCTX or treated daily with CsA 12 weeks after transplantation. No surviving mWGE grafts were found in any group, thus these bars are not shown

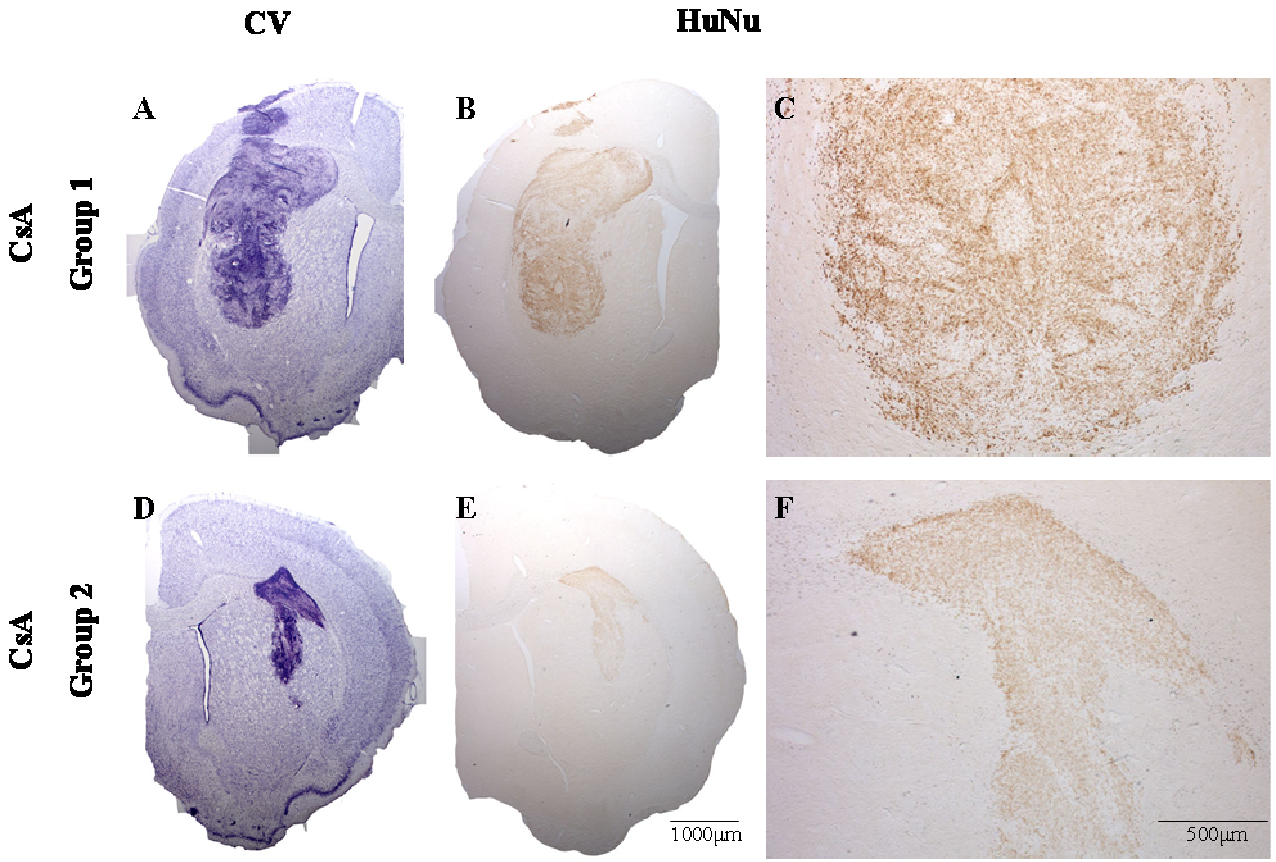


Figure 4.2 Representative surviving hCTX transplants in hosts treated daily with CsA from transplant groups 1 and 2 stained with CV (A,D) and HuNu (B,C,E,F). Transplants from group 1 (A-C) were found to be significantly larger than those from group 2 (D-F); F (1, 25) = 15.970 $p < 0.001$. Surviving human transplants were large in the majority of cases, in particular in group 1 transplants, which had a swirling appearance resembling overgrown, proliferating transplants

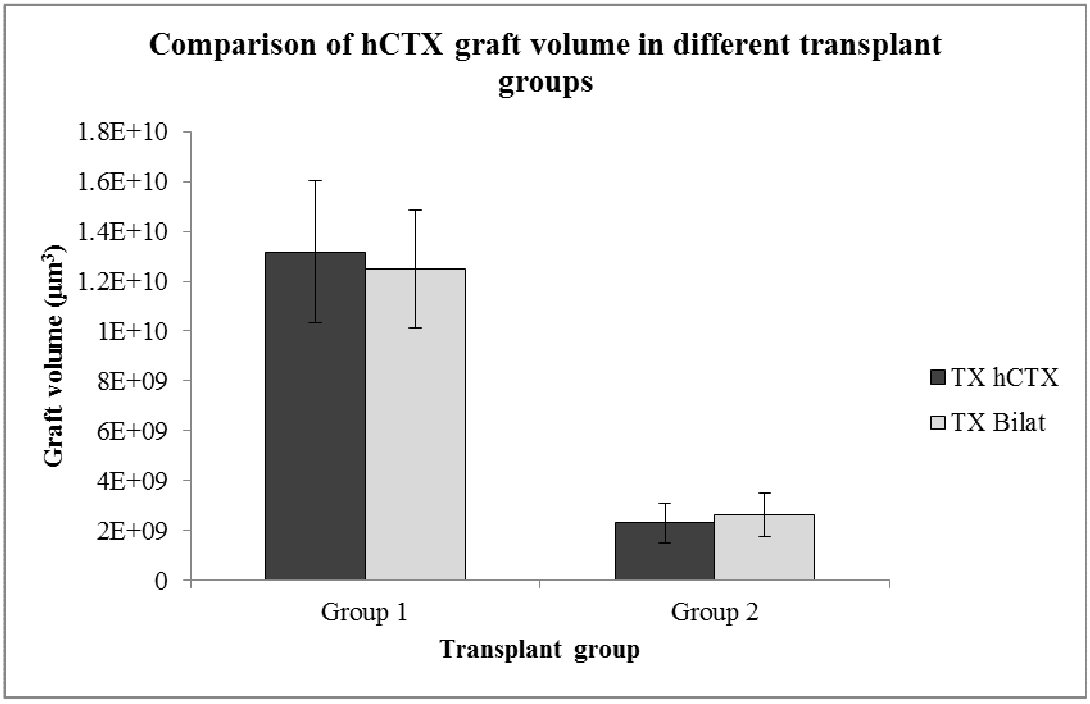


Figure 4.4 Graft volume of hCTX transplants in rat hosts at 12 weeks post-transplantation. Transplants were carried out on two separate days (Group 1 and Group 2) due to availability of human tissue. Hosts received either a unilateral transplant of hCTX, or bilateral transplants of both mWGE and hCTX

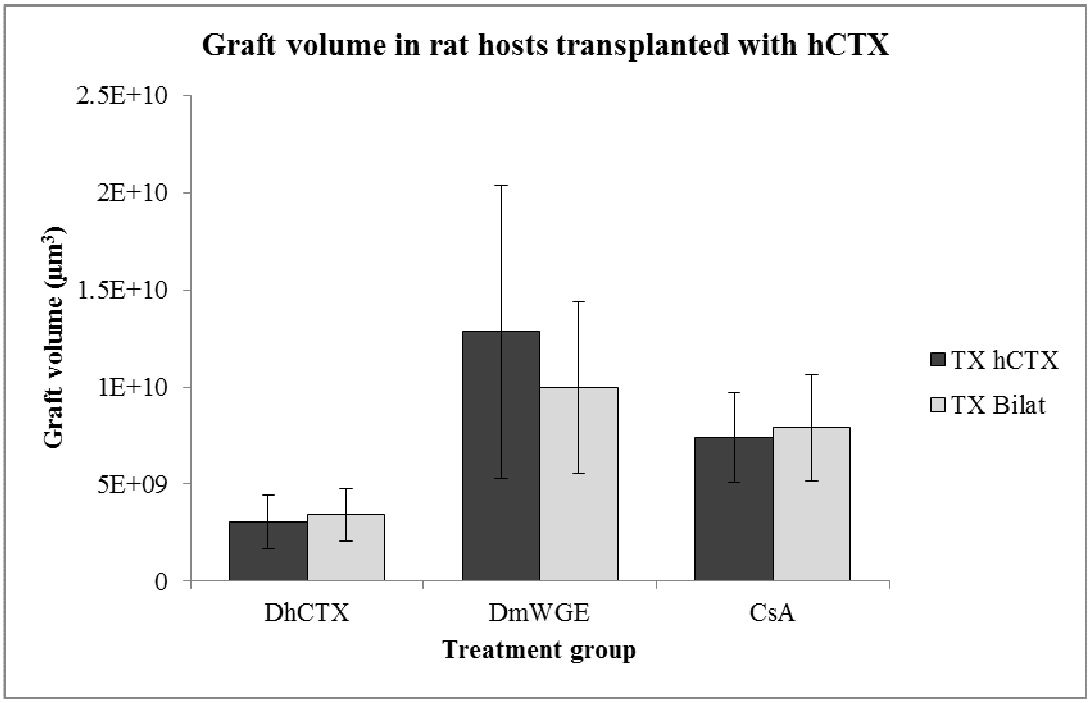


Figure 4.3 Graft volumes of hCTX transplants in rat hosts either desensitised with hCTX (DhCTX); mWGE (DmWGE) or treated daily with CsA. Hosts received either a unilateral transplant of hCTX, or bilateral transplants of both mWGE and hCTX.

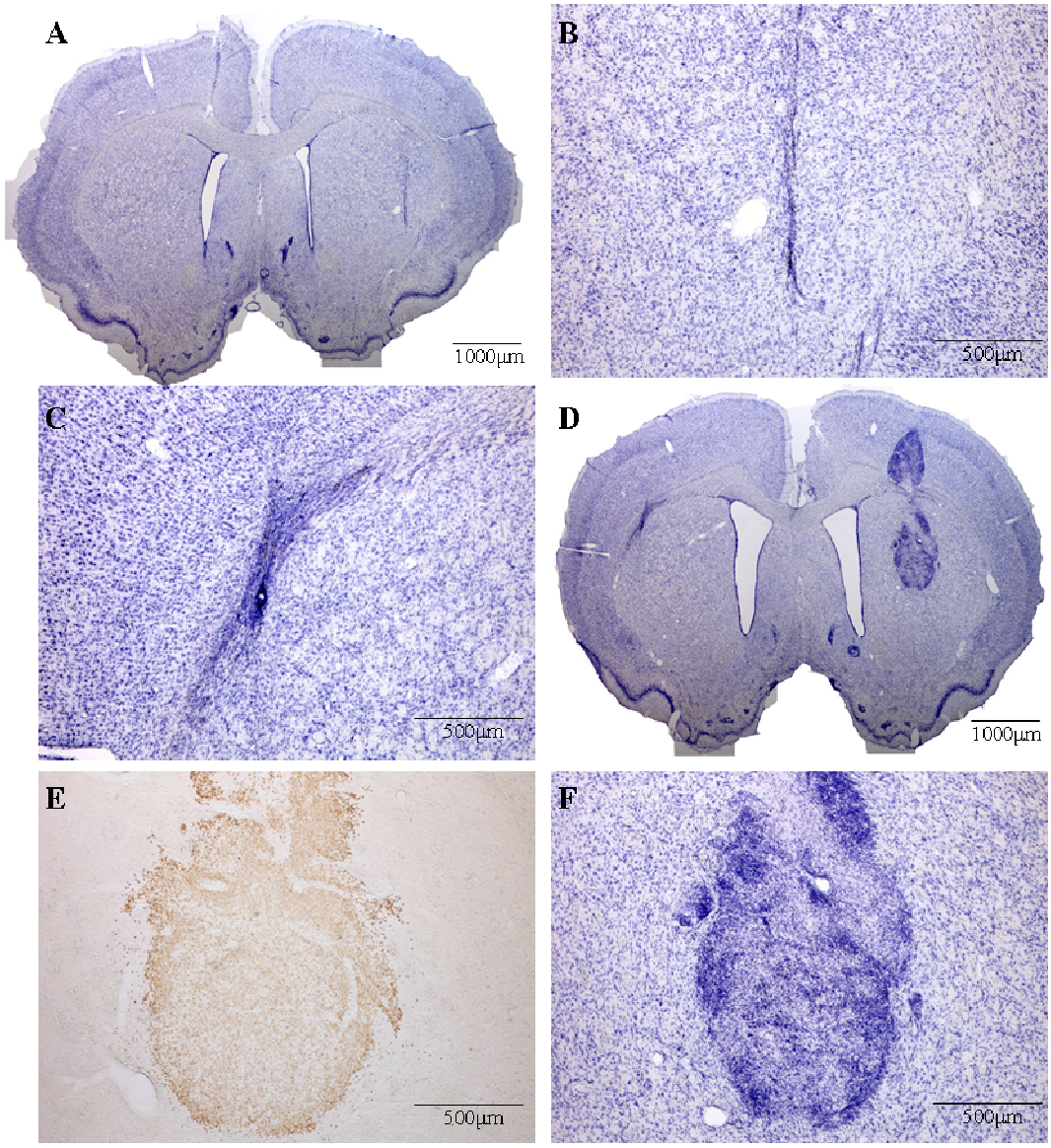


Figure 4.5 Photomicrographs of a CV stained section from a control host treated daily with CsA and transplanted with mWGE showing a lack of transplanted cells at 12 weeks, and only a needle tract remaining (A,B). A host transplanted bilaterally showing no mWGE graft in the left striatum (C,D) and a surviving hCTX transplant in the right striatum (D-F). All staining displayed is CV except for panel E which shows HuNu staining of surviving human cells. This staining was typical of mWGE and bilateral transplants.

4.4.2 Host immune response to transplants at 12 weeks

The immune response was assessed with immuno-staining for both $CD8^+$ and $CD4^+$ T cells, and the microglial marker CD11b (OX42). Stains were graded with the rating scale described previously, and differences assessed using non-parametric Kruskal-Wallis analyses of variance. No significant differences were found in any of the immune markers between the three transplant groups (DhCTX, DmWGE, and CsA), indicating that surviving transplants in desensitised hosts were likely to continue to survive comparably to those receiving daily CsA immune suppression; CD4: H (5) = 4.678, p = 0.456; CD8: H (5) = 6.494, p = 0.261; CD11b: H (5) = 4.416, p = 0.491. Examples of grading for each stain can be found in **Figure 4.6**. Although at 12 weeks post-transplantation no immune response may be expected, since the initial inflammatory response to transplants would have subsided and rejected transplants would no longer yield a response, these findings confirm that transplants in desensitised hosts are not undergoing chronic rejection in the host brain. **Figure 4.7** and **Figure 4.8** show the spread of immune marker grading for CD4, CD8 and CD11b in all transplanted hosts, highlighting the lack of variability between groups.

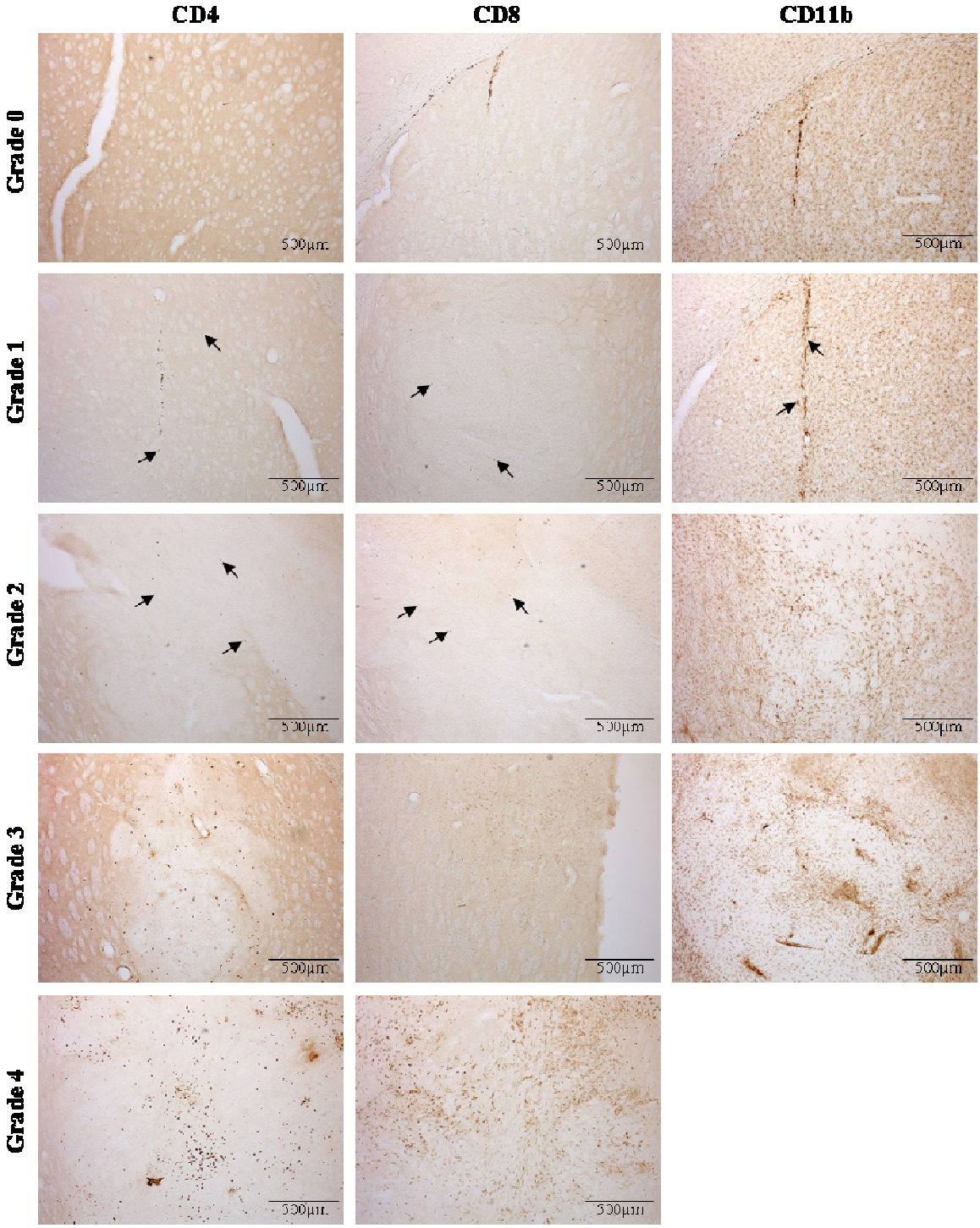


Figure 4.6 Examples of grades assigned to immune marker staining for $CD4^+$ and $CD8^+$ T cells and $CD11b^+$ microglia. No section was assigned a Grade 4 for microglial staining therefore no picture is shown. Grade 0 shows no staining in the grafted area, with only a scar from the needle seen. Increases in staining for each grade can be seen in and around the grafted area according to the rating scale described in **Chapter 2.** Arrows point to staining in lower grade images.

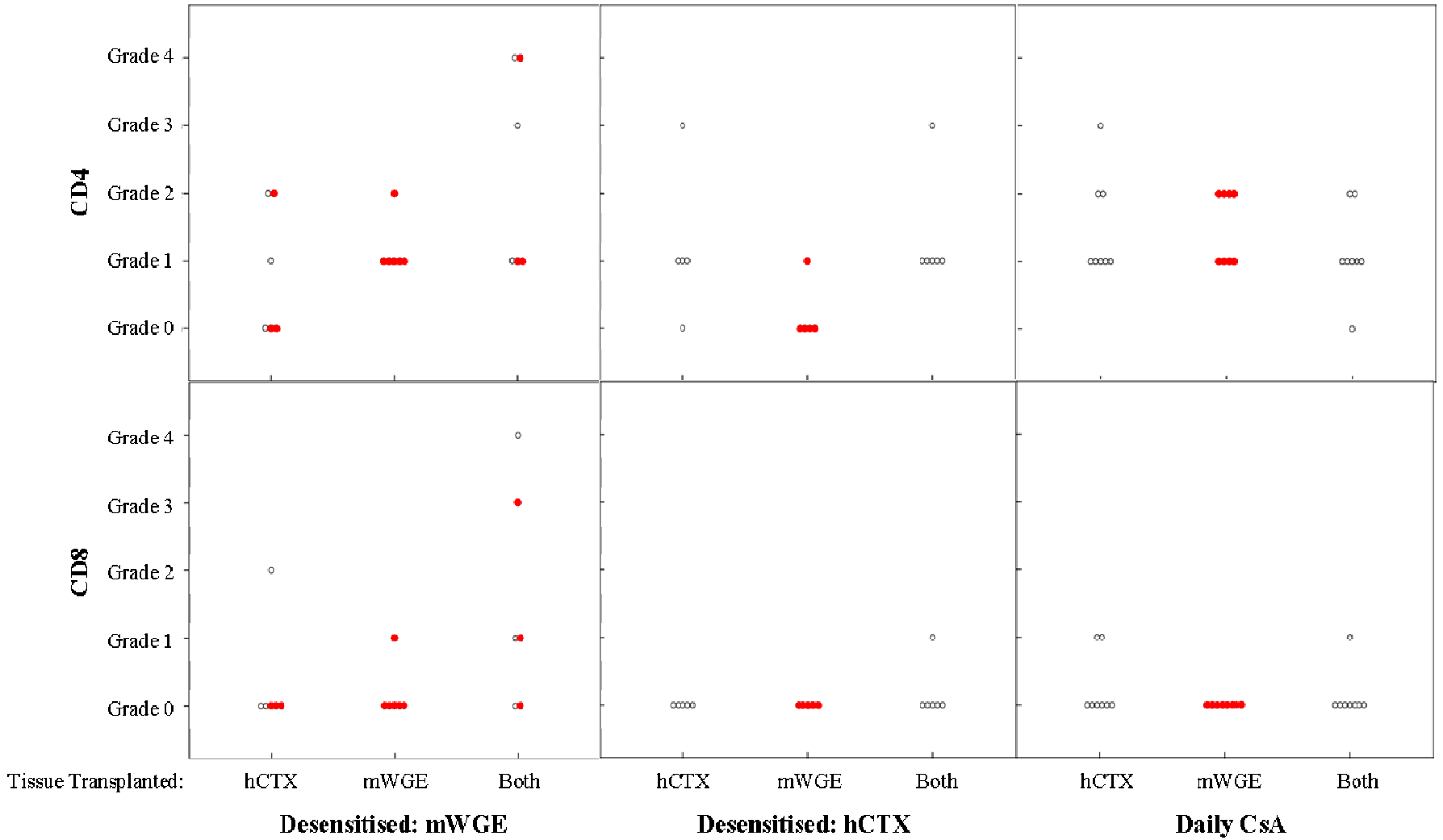


Figure 4.7 Gradings for CD4+ and CD8+ T cells in transplanted rat hosts at 12 weeks post-transplantation. Each circle corresponds to one host, with a red circle for rejected transplants. In the case of bilateral transplants, the grade is assigned to the side containing the hCTX graft since all mWGE transplants were rejected. Analyses were only conducted to compare surviving transplants.

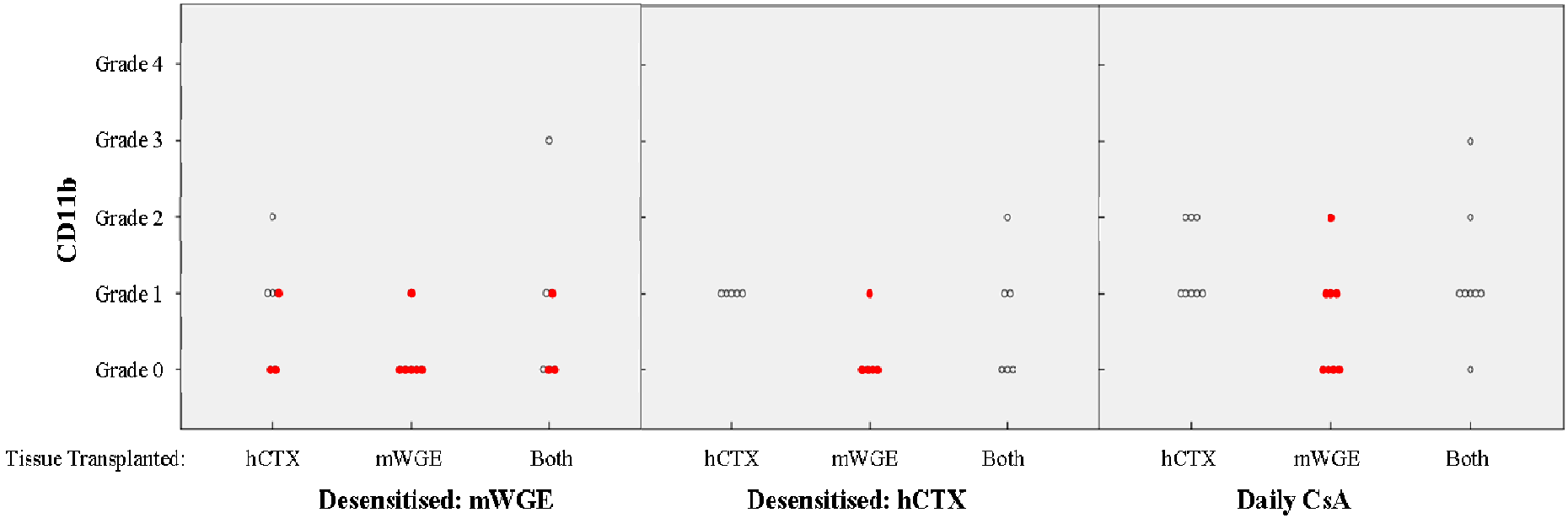


Figure 4.8 Grading for the microglial marker CD11b (OX42) in transplanted rat hosts at 12 weeks post-transplantation. Each circle corresponds to one host, with a red circle for rejected transplants. For bilateral transplants, the grade is assigned to the side containing the hCTX graft since all mWGE transplants were rejected. Analyses were only conducted to compare surviving transplants.

4.5 Discussion

The aim of the experiment described in this chapter was to determine whether desensitisation of the rat host to a neural transplant is specific to the species of the foetal donor tissue used; that is, do the cells used for desensitisation and neural transplantation need to be from the same species? Host rats were desensitised neonatally to either mWGE or hCTX, and a separate group of adult naïve rats were treated daily with CsA from one day prior to transplantation for the duration of the experiment. Subsequent transplants of hCTX or mWGE were delivered to the adult striatum, or a bilateral transplant of both tissue types. Good graft survival was found in human to rat transplant groups treated with CsA or desensitised to hCTX, confirming previous findings. However, contrary to the original hypothesis, more than 50% of hCTX transplants survived in host rats that had been desensitised with mouse tissue. The reason for this finding is not clear. Potentially some factors related to the neonatal inoculation may have rendered the host immune system more tolerant to transplants in general, and this would require further studies using additional interspecies transplants to resolve. Alternatively, certain surface antigens on the mouse donor tissue may bear sufficient similarity to the human tissue to prevent recognition of this transplant by the host immune system. An untreated control group, in which hosts receive a neural transplant but no immune suppression or neonatal desensitisation, was not included as many such controls have been included in previous studies and long term (> 4 weeks) survival has never been seen in a single case. However, given the unexpected nature of the result, it would be important to replicate this finding with the inclusion of a “no treatment” group to confirm that survival of hCTX in hosts desensitised to mWGE was due to this desensitisation rather than some other factor.

In hosts receiving bilateral transplants, the hCTX graft was found to survive in all hosts desensitised with hCTX or treated with CsA as well as in 50% of hosts desensitised with mWGE. In all of these cases large surviving grafts could be identified, of a comparable size to unilateral transplants, despite the simultaneous delivery of an mWGE graft, which was subsequently lost. Thus, the presence of tissue from a different donor species in the brain apparently had no effect on the survival of hCTX transplants. Furthermore, the subsequent loss of mWGE grafts

(through whatever mechanism, possibly rejection) did not cause rejection of hCTX. Previous reports have shown a second allogeneic or syngeneic CNS graft is likely to induce the rejection of both grafts, although this is in a situation in which grafts have been delivered sequentially (Widner and Brundin 1993), whereas in the current experiments grafts were delivered concurrently.

No surviving transplants of mouse tissue were found in any treatment group. This finding occurred not only in desensitised hosts but also in those treated with the conventional immunosuppression regime of daily CsA, which has previously been shown to promote survival of a mouse transplant (Pakzaban and Isacson 1994). Additionally, neural mouse to rat xenografts have been reported to survive previously even in the absence of immunosuppression (Brundin *et al.* 1985; Daniloff *et al.* 1985a; Daniloff *et al.* 1985b; Daniloff *et al.* 1984). The fact that mouse transplants were rejected even under immunosuppression suggests a problem with the donor tissue. This could be attributed to issues with the cell suspension used for transplantation, related to the donor tissue from that specific litter of mice. However since the control animals were transplanted in both transplant groups this is unlikely to be the case. Alternatively it is possible that there is a problem with the treatment of the cells for dissociation to a single cell suspension. Cell suspensions were prepared in these experiments using a standard 20 minute trypsin digest prior to trituration of the cells. There has been a suggestion that this treatment is too harsh for the mouse tissue (Breger, L., personal communication), and that a shorter digest in trypsin (e.g. 10 minutes) would improve the survival of tissue following transplantation. This raises questions about the preparation of mouse donor tissue for transplantation. If this was the case, then mWGE transplants were unlikely to have been rejected by the host immune system, rather the cells would not have survived the dissociation and transplantation process, in which case contralateral grafts of hCTX would not have been affected by rejection of mWGE grafts. To determine whether this was the case would require repeating the experiment and assessing immune marker staining at an early point after transplantation to characterise the immune response.

In this experiment, human donor tissue was derived from hPF CTX, whereas mouse donor tissue was from PF mWGE. Although ideally the same donor tissue

would have been used from each species, issues with the availability of human donor tissue meant that more hCTX was available for desensitisation and transplantation. Whilst hCTX tissue is more abundantly available than WGE for transplants investigating the properties of neonatal desensitisation, its use to transplant may not necessarily be appropriate to determine the survival of transplants since ultimately this will need to be applied to the relevant tissue type. The surviving human transplants both from the work in this chapter and **Chapter 3** were found to be very large. Investigation of this aspect by staining for the mature neuronal marker, NeuN, and the proliferative marker, Ki67 (see **Appendix 6**) showed the presence of immature, proliferating cells in the core of the graft. Further staining with doublecortin would be helpful, to confirm that these proliferating cells are of a neuronal origin, as opposed to connective tissue or meninges, which may have been dissected in error. Since the developing cortex would be highly proliferative, there is a possibility that the survival of transplants of this tissue may diverge from that observed with a different tissue type such as the WGE, and may explain why these transplants are more likely to survive in hosts even following desensitisation with tissue from a different donor species.

An additional finding from this study was a difference in graft volume of human transplants between hosts transplanted in the first group as compared to the second, with larger transplants found in group 1 than group 2. Cells transplanted in group 1 host animals were derived from a younger foetal donor (CRL 35.5mm) than those in group 2 (CRL 46.7mm). Since large differences were observed between the graft volumes from these foetal donors, this highlights the variability which may be observed dependent on the age of the donor foetal tissue. The effect of this on desensitisation and graft survival should be considered in future experiments. Due to the scarcity of human foetal tissue, donor tissue of a large age range has been used in experiments to investigate desensitisation and graft rejection, since there is no intention of assessing functionality of transplanted cells at this stage of the studies. However, this experiment and previous studies have suggested that younger donor tissue may result in better graft survival following transplantation to the CNS (Brevig *et al.* 2008). This has been demonstrated in both mouse to rat (Zimmer *et al.* 1988) and human to rat neural xenografts (Freeman *et al.* 1995), as well as being found in mouse to mouse transplants (see **Chapter 6)**. This has been attributed to the

lack of microglial precursor cells in younger donor tissue (Dalmau *et al.* 1997) which may act as donor APCs, since microglial precursors have been shown to express MHC antigens and co-stimulatory molecules *in vitro*, with the ability to stimulate naïve T cells (Frei *et al.* 1987). Since the appearance of larger transplants was different to the smaller ones, in that they had a swirling appearance in staining with CV similar to overgrowing transplants, there is the potential that this is due to differences in dissection between the two donor embryos, in addition to the difference in age.

4.6 Conclusions and Future Work

Due to the lack of survival of mWGE transplants and the need for an untreated control group it cannot be definitively concluded as to whether desensitisation is specific to species type. However, this experiment did show survival of at least half of hCTX transplants in hosts desensitised with tissue from a different donor species (mWGE). This finding requires further investigation to determine whether this outcome is due to desensitisation to an epitope which spans both human and mouse, or whether this is due to a more general phenomenon, including comparisons with untreated controls and with transplants of WGE rather than CTX. Therefore this experiment requires replication with this additional control group and modifications to the preparation of mouse donor tissue to aid in survival of transplants.

A second experiment is currently being planned in order to determine the donor specificity of desensitisation to xenogeneic tissue and, in addition, to determine whether tolerance to neonatally injected tissue extends beyond the CNS. This could provide evidence for the induction of tolerance via an established skin graft method as opposed to a CNS transplant which may be considered to be a more protected environment than the periphery and thereby promoting survival. It would also be possible to use the skin grafting method as an immune challenge for neural grafts. Previous studies have shown that if a peripheral skin graft if the same donor tissue is applied to an animal which already has a surviving neural graft of tissue from the same species (rat to rat), rejection will occur (Duan *et al.* 1997). It would be interesting to determine whether any tolerance induced through neonatal desensitisation is strong enough to prevent this phenomenon. In particular since it has been shown that the acceptance of neural grafts transplanted into rat neonates is not stable and that a peripheral challenge, such as a skin graft, induces rejection at later time-points (Pollack and Lund 1990). These experiments were not possible for the purpose of this thesis due to the necessary animal licence amendments required needing Home Office approval which was not granted within sufficient time to conduct the experiment.

The findings from this chapter have raised questions about whether the standard tissue dissociation method used to prepare donor cells for transplantation is too harsh for use with mouse tissue. This is addressed to some extent in **Chapter 6** and is also

the subject of further systematic studies to examine the effect of various treatments with trypsin and trituration of mouse WGE on the viability of cells, their phenotype *in vitro* and following transplantation *in vivo*. Such information will inform the design of future experiments to further explore the specificity of desensitisation.

Chapter 5

Can mouse hosts be desensitised to human foetal tissue transplants?

5.1 Summary

The experiments discussed in this chapter were designed to investigate whether mouse hosts could be successfully desensitised neonatally to human primary foetal (hPF) neural tissue to allow survival of a neural xenograft in adulthood. Previous work in this thesis and from this lab group has demonstrated successful long term xenograft survival in rat hosts as a result of desensitisation following an i.p. injection of hPF cells in the early neonatal period. It is important to determine whether this may be achieved across species and in transgenic mouse models of disease. These experiments use a QA lesion model of HD in CD-1 mice neonatally desensitised to human neural tissue, treated with CsA, or untreated. Hosts received striatal transplants of human or mouse neural tissue to determine whether grafts could survive for up to 12 weeks post-transplantation[†].

Experiment 1 compared survival of human xenografts and mouse grafts in desensitised and untreated mouse hosts. As poor survival was seen in all groups, **Experiment 2** included a CsA treated control group, however a lack of transplant survival was still observed universally. Desensitisation and transplantation protocols were modified in **Experiment 3**; increasing the number of cells transplanted, and reducing the time between lesion and transplant. Varying numbers of cells were used for desensitisation, and transplant survival was compared with immunosuppressed hosts. As initial staining suggested good survival in mice desensitised with the greatest number of cells, **Experiment 4** tested even higher cell numbers. However, further investigation revealed poor survival of human cells in the majority of hosts. This has made it impossible to assess the desensitisation method in mice, but has highlighted a general problem with mice as transplant hosts, addressed in **Chapter 6**.

[†] The experiments described in this chapter are published in NeuroReport (Roberton *et al* 2013). The compilation of data reported in the Combined Results section was carried out by David Harrison (DJH) for this manuscript.

5.2 Introduction

Preclinical testing of human donor cells requires transplantation into rodent models of disease in order to assess functional efficacy and confirm safety. Lesion models of both PD and HD may be used, allowing behavioural assessment of a deficit post lesion to measure any improvement following transplantation. Neurotoxic lesion models can replicate some features of disease including relevant cell loss and histological changes, resulting in some disease related behavioural deficits. Genetic models can provide a more relevant model of some aspects of the neuropathological features of a disease, but tend to represent its more extreme forms and can also be difficult to standardise. However, in neurodegenerative disease, genetic models can more accurately mimic the progressive nature of disruption to particular circuitry in the brain. This is relevant in the study of cell transplantation in order to identify whether this progressive disease process also affects the development and survival of transplanted cells (Nakao and Itakura 2000).

For a number of years, efficient genetic modification has been established in mice, resulting in the development of thousands of genetically modified mouse strains, including models of neurodegenerative disease. Several HD models are well established, containing insertions of either the full length HD gene, or the expanded CAG repeat fragment as discussed in **Chapter 1**; section 1.1.2, and long term assessment of pathology and behavioural phenotypes of a number of these mouse lines using an established battery of tests of motor and cognitive deficits is underway (for reviews see Brooks and Dunnett (2013); Brooks *et al.* (2012)). Although a number of Tg rat models of neurodegenerative diseases now also exist including models for HD (von Hörsten *et al.* 2003) and PD (See Welchko *et al.* (2012) for a review), the technology for ESC-based gene-targeting in rats is relatively immature in comparison (Zheng *et al.* 2012). Therefore, mouse models of disease currently exist in greater numbers and have been more extensively examined than those created to date in rats.

The ability to test human donor tissue in such mouse models of disease would open up a variety of Tg tools. For example, investigating the effect of transplanting cells into a degenerating environment, as in the case of HD, where differences have been observed in immune system activation and inflammation in patients as well as

in Tg models (Björkqvist *et al.* 2008; Ellrichmann *et al.* 2013; Kwan *et al.* 2012). Mouse models with modifications to the immune system exist in abundance in comparison to the rat, thus achieving desensitisation of mice would allow the use of these to elucidate the mechanisms underlying the method.

As in human to rat grafts, there is a need to circumvent xenograft rejection; therefore the use of neonatal desensitisation to avoid conventional immune suppression would permit full behavioural assessment of transplant function and a long term assessment of the cells *in vivo* to determine whether they are also affected by the degenerative disease process. As yet neonatal desensitisation has only been validated using rats. Although the desensitisation method has been validated by other groups in rat transplant experiments (Singhal *et al.* 2012; Zhang *et al.* 2013), there is a suggestion that it does not translate well to the mouse (Janowski *et al.* 2012; Mattis *et al.* 2014). Studies investigating mouse desensitisation have used a range of different cell types in different transplant paradigms into different strains and species. This suggests the utility of the method could vary dependent on host or donor tissue type, and therefore highlights the importance of adequate validation with systematic analyses of these variables.

The primary aim of the experiments described in this chapter was to determine whether neonatal desensitisation to neural tissue in mice is sufficient to allow survival of a human neural transplant in adulthood. All experiments described in this chapter were carried out in QA lesioned CD-1 mouse hosts. This strain was chosen as their large litter sizes provide sufficient animal numbers for transplant experiments. Additionally, infanticide levels in this strain are low, allowing brief separation of pups from the mothers for a neonatal injection without resulting in a loss of host animals. Although the CD-1 strain provides a good host for transplantation, the majority of genetic models of disease are bred onto a BL/6 background, and a larger body of work has been carried out in the behavioural characterisation of this strain. Therefore although we aim to seek ‘proof of principle’ for desensitisation and transplantation protocols in CD-1 mice, ultimately the aim would be to successfully transplant into BL/6 hosts and models of disease. An additional pilot experiment described in **Appendix 7** attempted to investigate the potential for neonatal desensitisation in this strain.

This chapter includes findings from four mouse transplant experiments investigating; *i)* survival of transplants to the QA lesioned and intact striatum of human and mouse foetal tissue in desensitised and naive mouse hosts; ii) survival of striatal transplants of human and mouse tissue in desensitised, CsA treated and untreated mouse hosts; *iii)* survival of human xenografts in mice treated with CsA or desensitised with three different cell numbers of human foetal tissue, and; *iv)* survival of human xenografts in mice tolerised with increasing numbers of human cells. Experiments are described sequentially with a discussion of the outcomes of each, and how this drove the design of the subsequent experiment, with a final discussion of the findings and conclusions from this chapter. Successful desensitisation of mouse hosts could not be demonstrated despite a number of modifications to the desensitisation and transplantation protocols, and attempted validation in a second strain. However, problems were identified with transplant survival even in immune suppressed hosts and those receiving grafts of mouse tissue. This is suggestive of a problem with the mouse as a neural transplant host.

5.3 General experimental design

The basic design of all experiments discussed in this chapter was the same, with minor modifications depending on the specific aims of each experiment. **Figure 5.1** below outlines the standard design of these studies.

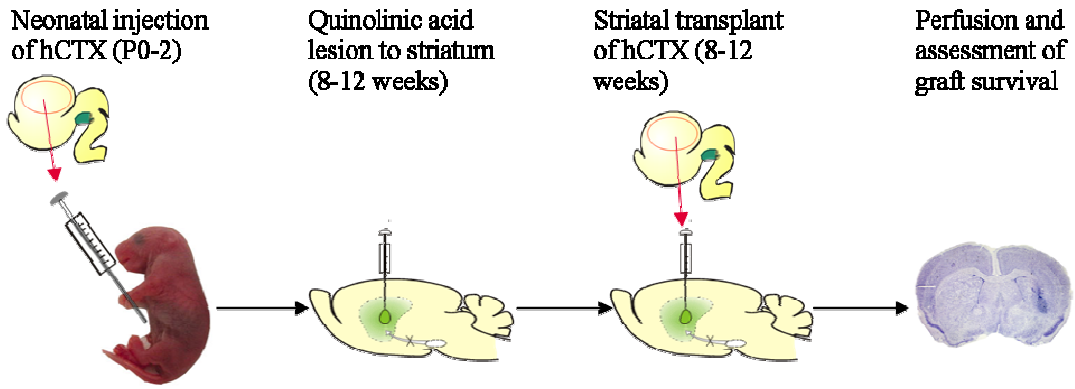


Figure 5.1 Outline of the basic design of mouse desensitisation and transplantation experiments

5.3.1 Dissection and dissociation of hCTX and mWGE

For xenografts; hPF tissue (8-12 weeks post-conception) was collected from mToP and CTX dissected. For mouse tissue grafts; E14 CD-1 mouse foetuses were collected and WGE dissected. All tissue was dissociated and prepared as cell suspensions. Viability greater than 75% was considered sufficient for tolerising and 85% for transplantation.

5.3.2 Neonatal desensitisation and adult transplantation

To induce desensitisation; mice were injected neonatally with a suspension of hPF CTX from postnatal day 0-2 (P0-2) with the exception of the first experiment *(i)* where mice were injected at P3 due to lack of availability of human tissue. In standard experiments mice were injected with 1µl of suspension containing $1x10^5$ cells in DMEM/F12. In experiments testing greater cell numbers animals were injected with the total number of cells specified. In the case of an injection of greater than $5x10^5$ cells, suspensions were administered in a volume of 2µl.

As determined by experimental design, adult CD-1 mouse hosts either received a unilateral QA lesion to the right striatum, or the host tissue was left intact. According

to experimental group, two days to two months later adult mouse hosts received transplants into the intact or lesioned striatum of hCTX or mWGE to a total of $3x10^5$ or $5x10^5$ cells in 2µl. Survival of transplants was assessed 6 to 12 weeks after transplantation

5.3.3 Quantification and statistical analyses

Positive graft survival was determined through assessment of CV staining of 1:12 sections by two independent assessors blind to condition. Where possible, survival of transplants was confirmed with further immunohistochemical staining of HuNu (for xenografts) and a mature neuronal marker (NeuN) for all sections. Due to loss of tissue sections in storage at 4^oC the host immunological response to xenografts could only be assessed for Experiment *iv)* (see **Appendix 5** for details). Iba1 microglial staining was graded according to the rating scale detailed in **Chapter 2**.

5.4 Experiment *i)* Striatal grafts of human and mouse tissue in untreated and desensitised mouse hosts

5.4.1 Experimental Design

A total of 32 (15 female, 17 male) CD-1 mice were either "desensitised" or "untreated" and received a transplant of hPF CTX ($3x10^5$cells) or E14 mWGE ($3x10^5$cells) into the intact or QA lesioned right striatum from 8 weeks of age (weighing >20g). Mice were desensitised (n=16) neonatally with an i.p. injection of $1x10^5$ hCTX cells at postnatal day 3 (P3). Untreated mice (n=16) received no neonatal injection and were not treated with any other immunosuppression throughout the experiment. Two groups of untreated and desensitised animals (n=4 per group) were not transplanted and were kept as lesion only controls. Mice were perfused at 6 to 12 weeks post transplantation and brains taken for assessment of graft survival (**Table 5.1**).

Group	Treatment	Lesion	Transplant	Time post-transplantation	Number of Mice
1	Untreated	QA	hCTX	6 weeks	4
				12 weeks	4
2	Desensitised	QA	hCTX	6 weeks	4
				12 weeks	3
3	Desensitised	None	hCTX	6 weeks	3
				12 weeks	2
4	Untreated	QA	mWGE	8 weeks	4
5	Untreated	QA	None	N/A	4
6	Desensitised	QA	None	N/A	4

Table 5.1 Mouse hosts included in each condition for Experiment *i)*

5.4.2 Results

To identify surviving grafts in transplanted hosts, sections from all animals were first stained with CV. Confirmation of transplanted human cells was achieved using immunohistochemical staining for HuNu. Staining of Nissl bodies with CV showed dense staining in the grafted area in a few animals, suggestive of surviving human grafts (**Figure 5.2**; A, B, E, and F). However, the majority was found in the immediate vicinity of the needle track resembling scarring rather than a larger, healthy looking graft (**Figure 5.2**; A and E). Comparable staining with CV could also be seen in animals which received lesions only and no transplants (**Figure 5.2**; C, D, G and H). Subsequent staining with HuNu did not clearly show positive staining of human cells, thus poor graft survival was observed across all groups. One animal appeared to clearly have a surviving graft in CV sections (**Figure 5.2**; B, F) but HuNu showed high background staining causing difficulty in the detection of specific positive staining of human cells (**Figure 5.3**). Additional stains could not be carried out due to contamination and degradation of all tissue sections stored in TBZ at 4^{o}C (See **Appendix 5**).

Surviving grafts were counted based on careful examination of CV staining due to unreliability of HuNu staining and are outlined in **Table 5.2**. Due to problems with HuNu staining and a lack of specific antibody or cell label for mouse grafts, it is possible that numbers of surviving grafts are overestimates. **Figure 5.4** shows examples of CV staining in hosts receiving transplants of mouse tissue, including the only surviving transplant (A). This surviving transplant was small and thin in appearance and could not be definitely demonstrated to contain surviving transplanted cells due to the lack of specific label. Despite the potential for overestimation of surviving transplants, only very few were identified, therefore no statistical analyses were carried out.

Treatment	Lesion	Transplant	Weeks post TX/			Total	%
			6	8	12		
None	QA	hCTX	0/4		1/4	1/8	12.5
Desensitised	QA	hCTX	1/4		1/3	2/7	28.5
Desensitised	None	hCTX	1/3		0/2	1/5	20
None	QA	mWGE		1/4		1/4	25

Table 5.2 Surviving transplants in mouse hosts from Experiment *i)*

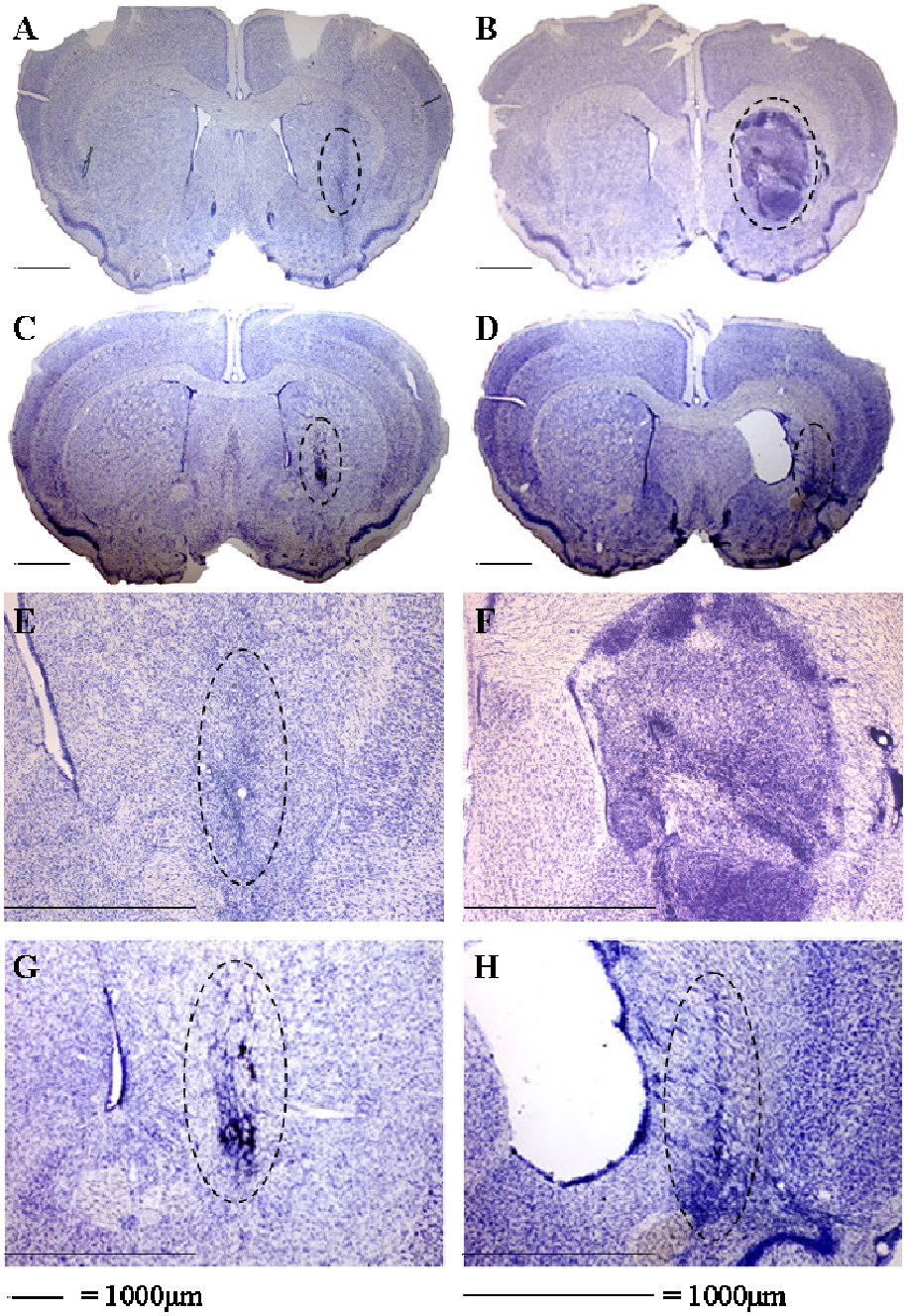


Figure 5.2 Photomicrographs of CV stained sections from mice tolerised with hCTX and transplanted with hCTX into the unlesioned striatum at 12 weeks post -transplantation (A, B, E, F), and lesion only controls (C, D, G, H). Staining that can be seen in animals with no transplants (G,H) was comparable to that in some transplanted animals (A), suggestive of only a needle tract. Staining in one host animal showed a clear large surviving graft, which had filled the striatum but did not seem to integrate, pushing into the ventricle (B, F).

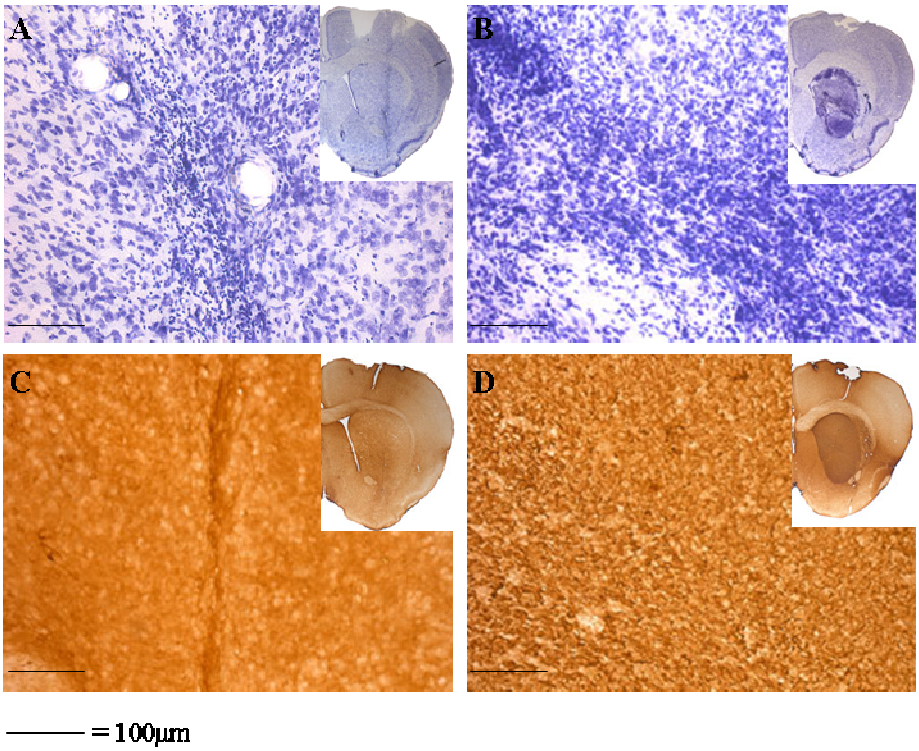


Figure 5.3 Staining for CV (A, B) and HuNu (C, D) in sections from mice desensitised and transplanted with hCTX (unlesioned). Lower power images are inset. HuNu staining showed a high background, making it hard to distinguish positive staining of grafted cells.

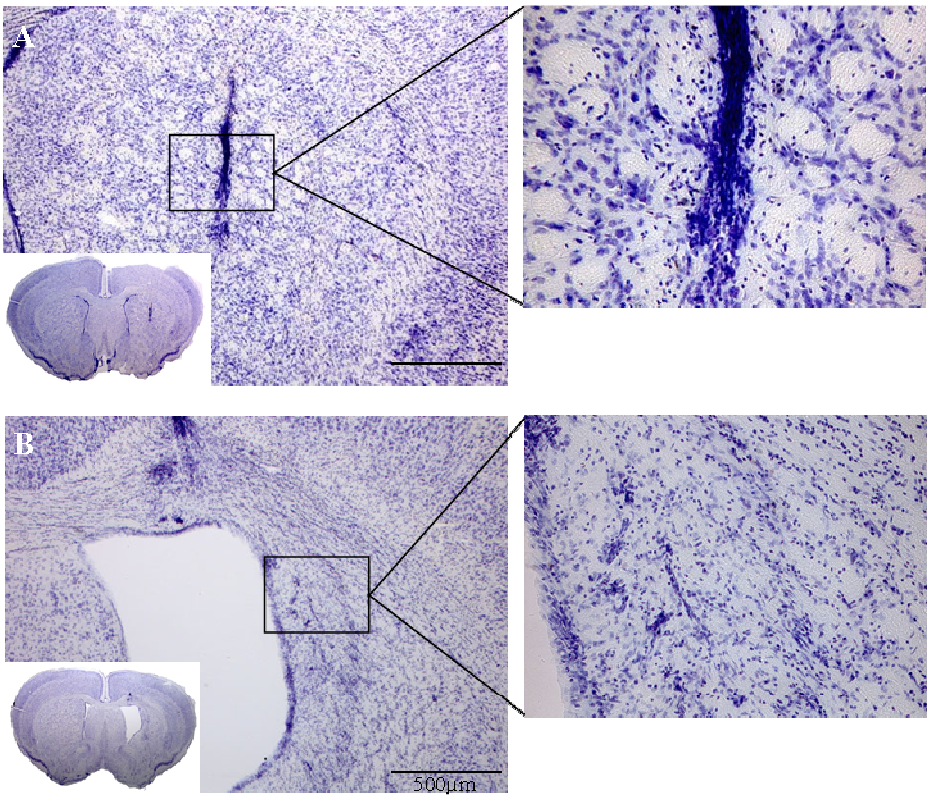


Figure 5.4 Photomicrographs of CV stained sections from mice transplanted with mWGE. A) shows a small potential surviving mouse transplant, and B) shows a transplant which has been rejected, with no clear surviving graft. Lower magnification images are inset, and higher magnification is displayed to the right

5.4.3 Discussion

Low numbers of surviving transplants were identified in Experiment *i)* through CV staining, however these could not be confirmed with HuNU staining. One transplant in a host tolerised with hCTX appears to have definitely survived to 12 weeks post transplantation, as a large dense area of staining can be seen on Nissl stained sections which is comparable to that seen in rat transplants. However, positive HuNu staining in these sections could not be verified as staining is too dark to distinguish from background. Additionally, survival of mouse transplants could not be confirmed due to the lack of marker for mouse donor cells. Although staining with NeuN and DARPP-32 (MSNs) in a QA lesioned mouse brain may have revealed the presence of a surviving graft, this was not possible due to the loss of tissue sections as discussed in **Appendix 5**. Therefore although one surviving mouse graft was identified in this experiment based on comparison with previous surviving mouse transplants (Precious, SV. unpublished data; Kelly *et al.* (2007)) it is not possible to confirm this with CV staining alone due to this lack of definitive labelling. This surviving graft was found to be thin and pencil-like in appearance with the only staining possibly representing transplanted cells to be close to the needle track and cell deposit rather than showing graft development and integration into the host tissue as may be expected in rat tissue transplants.

A number of problems can be identified from the findings of Experiment *i).* The lack of an immunosuppressed control group (e.g. treated with CsA) does not allow determination of whether human grafts would have survived if hosts were treated with a conventional immunosuppressant as compared to desensitised hosts. However, the fact that survival of mouse tissue was also found to be poor suggests that a xenograft would be most likely rejected. Additional problems are evident in the identification of surviving grafts. CV staining of Nissl bodies appears to be an unreliable method alone of detecting surviving grafts, since staining in these sections was not distinguishable from lesion only controls and grafts which were possibly rejecting. In this instance; staining with a human specific antibody proved problematic due to high background staining. This is likely due to cross-reactivity of the anti-mouse secondary antibody used. Sections in this experiment also appear underfixed, suggestive of a problem with perfusion. This also may be related to the

deterioration of remaining sections which has prevented subsequent staining of stored brain sections. Therefore numbers of surviving transplants may be overestimates due to the lack of a definitive marker for transplanted cells.

The findings of this experiment prompted the design of Experiment *ii)* in which the survival of human xenografts in tolerised hosts was compared to survival in CsA treated and untreated hosts. Since Experiment *i)* showed poor survival; a CsA treated group was included to identify whether poor survival of xenografts was due to a lack of successful desensitisation, or poor transplant survival overall even in immune suppressed hosts. As mouse graft survival was also poor; an untreated and CsA treated mouse transplant group were included to determine whether immune suppression would promote survival of an allo-transplant of mWGE.

5.5 Experiment *ii)* Striatal mouse and human grafts in untreated, desensitised, and CsA treated mouse hosts

5.5.1 Experimental Design

A total of 51 CD-1 mice were included in this experiment (25 male and 26 female). 20 mice were injected neonatally with $1x10^5$ cells (hCTX) with remaining animals receiving no neonatal injection. At 6-8 weeks of age all mice received unilateral quinolinic acid lesions to the right striatum. Grafts of hCTX or mWGE ($3x10^5$ cells) were then transplanted into the lesioned striatum of desensitised and untreated mice. Those mice that were not desensitised were treated daily with CsA starting from the day prior to transplantation, or received no treatment. Graft survival was assessed at 6 weeks post-transplantation for all groups, with an additional desensitised group kept until 10 weeks post transplantation. The design is detailed in **Table 5.3** below.

Group	Treatment	Transplant	Time post-transplantation	Number
1	Desensitised	hCTX	6 weeks	10
			10 weeks	10
2	CsA	hCTX	6 weeks	11
3	Untreated	hCTX	6 weeks	5
4	CsA	mWGE	6 weeks	7
5	Untreated	mWGE	6 weeks	7

Table 5.3 Treatment and transplant conditions for mice in Experiment *ii)*

5.5.2 Results

Numbers of surviving transplants based on CV staining in all groups are outlined in **Table 5.4**. As with Experiment *i)* some dense staining was seen in the graft area, mainly as a scar or needle track in all transplant groups (**Figure 5.5**, **Figure 5.6**). In mice receiving grafts of mWGE tissue a number of surviving transplants were identified from CV staining (5/7), however these were small pencil grafts and could potentially have been rejected transplants (**Figure 5.5**). Transplants of mouse tissue delivered to hosts treated with daily CsA did not yield improved survival, in fact survival was found to be lower than those in untreated hosts (1/7 - **Table 5.4**)

In hosts receiving xenografts of hCTX tissue; HuNu immunohistochemistry in desensitised and CsA treated xenografted hosts revealed positive staining in only three grafts (**Table 5.4**; **Figure 5.6**); two in desensitised animals, and the other in a host treated with CsA. In CsA treated animals, a number of sections showed dense staining with CV, however the majority resembled rejecting transplants with vascularisation and often damage to the tissue (**Figure 5.7**)

Treatment	**Lesion**	**Transplant**	**Number of surviving grafts (weeks post TX)**		**Total**	**%**
			6 weeks	**10 weeks**		
Desensitised hCTX	QA	hCTX	2/10	1/10	3/20	15
CsA	QA	hCTX	7/13	-	7/13	54
None	QA	hCTX	1/5	-	1/5	20
CsA	QA	mWGE	1/8	-	1/8	13
None	QA	mWGE	5/7		5/7	71

Table 5.4 Surviving transplants in mouse hosts from Experiment *ii)* based on CV and HuNu staining of sections.

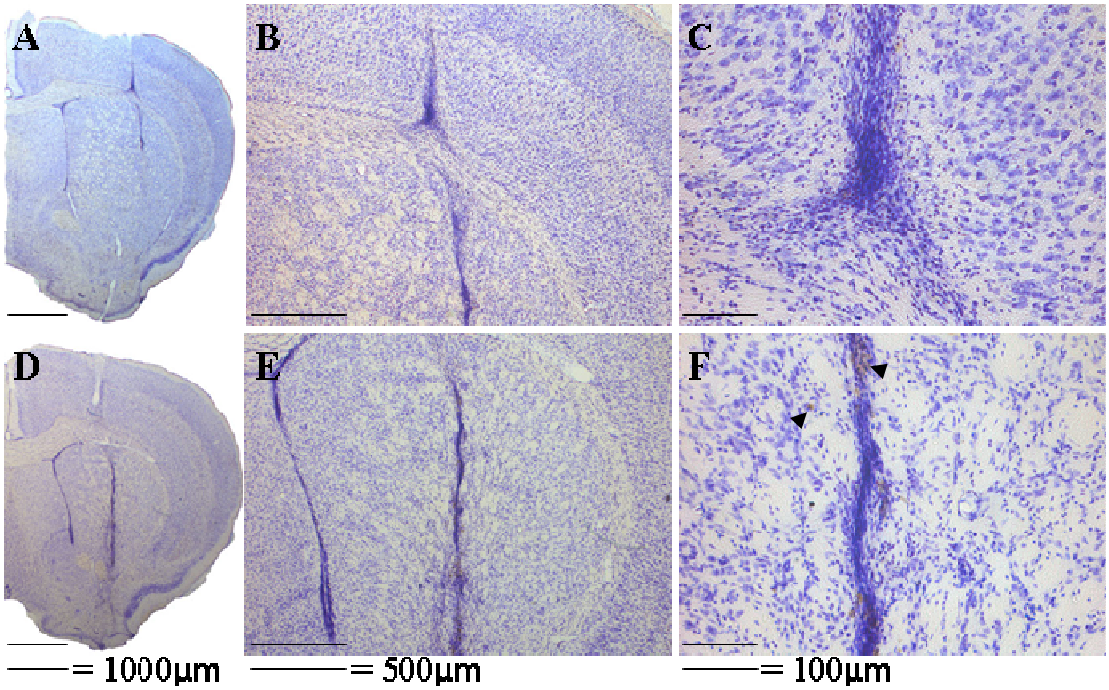


Figure 5.5 CV stained sections from mouse to mouse striatal grafts in two untreated hosts (A-F). Grafts are small and pencil-like in structure, possibly only representing rejected cells. Deposits of haemosiderin could be seen in a number of grafted areas (arrowheads in F).

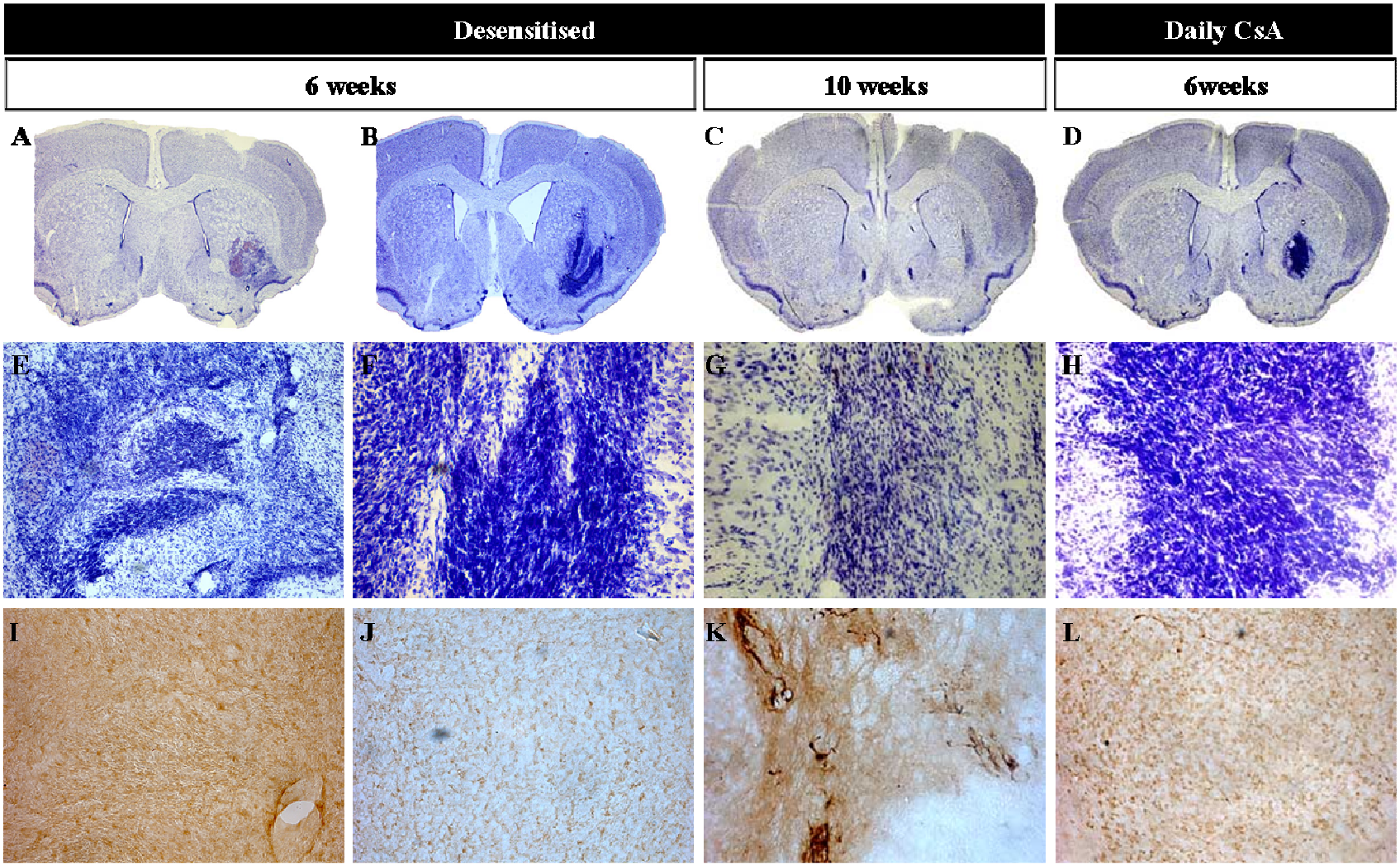


Figure 5.6 Human to mouse xenografts in hosts desensitised and transplanted with hCTX at 6 (A, B, E, F, I, J) and 10 weeks post transplantation (C, G, K) or treated daily with CsA at 6 weeks post transplantation (D, H, L). Sections are stained with CV(A-H) and HuNu (I-L). Positive staining could only be seen in three surviving transplants and was very hard to distinguish from background (I,J,L). Staining resembling transplants on CV sections did not always result in positive staining with HuNu (G,K)

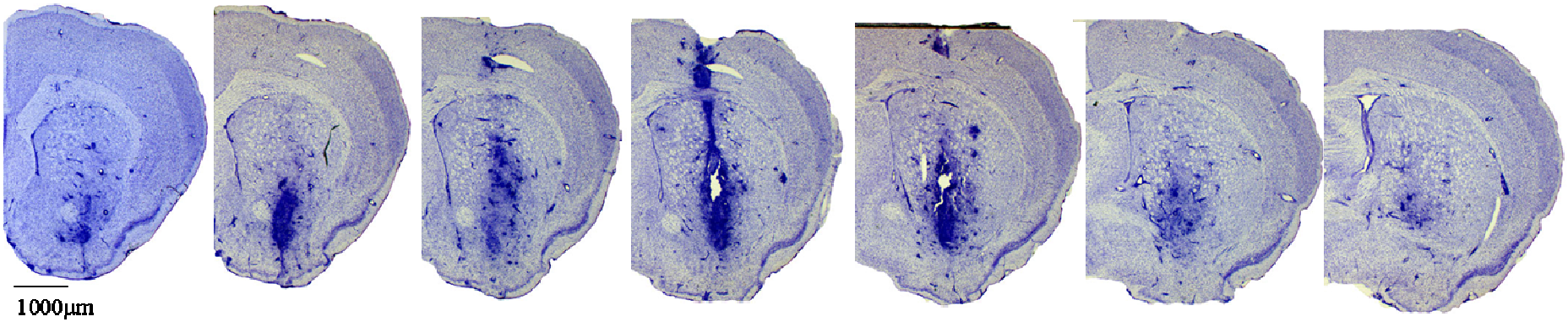


Figure 5.7 Photomicrographs of CV stained sections from a mouse host transplanted with hCTX and treated daily with CsA. CV staining resembles rejecting transplants with badly vascularised and necrotic appearing dense staining in the grafted area

5.5.3 Discussion

Experiment *ii)* was designed to compare the survival of xenografts in desensitised mouse hosts to those treated daily with CsA. As problems have been experienced previously in graft survival in mice (Experiment *i)*), immune suppressed controls transplanted with hPF cells were included to ensure any lack of survival was not due to the grafting protocol itself. Therefore the aim was to determine whether these parameters could be used to induce successful desensitisation to hPF tissue in mouse hosts. Additionally due to poor survival of mouse grafts in Experiment *i)*, two groups of host animals in this experiment also received transplants of mWGE, one of which received daily treatment with CsA.

Results from CV staining in sections from animals in xenograft groups suggested some survival of grafts in desensitised hosts as well as those treated with CsA. However, positive human specific staining (HuNu) could only be confirmed in a few. In view of these problems, in future experiments staining of immune markers is required to determine whether dense CV staining is observed due to an infiltration of immune cells as opposed to surviving transplanted tissue. Unfortunately this could not be carried out in this case due to degradation of tissue as described previously (**Appendix 5**). In mouse tissue transplant groups some survival was seen, however as in Experiment *i)* grafts were very small resembling needle tracks. Additionally these thin grafts appeared unhealthy and possibly rejecting, with deposits of haemosiderin clear on CV stained sections. CsA treatment did not improve the survival of mouse grafts in this experiment, with only one surviving transplant in this group.

Since previous successful neonatal desensitisation has been shown only in the SD rat, it is possible that the method may need to be modified for successful desensitisation to be achieved in the mouse. Furthermore, poor survival of mouse tissue transplanted to the mouse brain suggests the grafting protocol itself may also require modification. The remaining experiments in this chapter address modifications to desensitisation protocols, by testing whether using an increased number of cells for desensitisation of hosts improves survival of subsequent transplants. As all previous successful studies have been carried out in the rat, it is possible that a different optimum cell number is required.

5.6 Experiment *iii)* Striatal xenografts in mouse hosts treated with CsA or desensitised with varying numbers of hPF cells

5.6.1 Experimental Design

CD-1 mice (40) were injected neonatally with $1x10^4$; $1x10^5$ or; $5x10^5$ cells (hCTX) or received no injection. At 6-8 weeks mice received unilateral quinolinic acid lesions to the striatum and were grafted 8 days later with $5x10^5$ cells (hCTX). The fourth group of animals was treated daily with CsA starting from the day before transplantation. Half of each group was taken at 6 weeks post-transplantation to assess graft survival. Remaining animals were kept until 12 weeks post-transplantation (**Table 5.5**)

P0-2	hCTX tissue ($1x10^4$cells) injected i.p. (n=6)	hCTX tissue ($1x10^5$ cells) injected i.p. (n=11)	hCTX tissue ($5x10^5$ cells) injected i.p. (n=11)	No neonatal injection. Treated daily post-TX with CSA (n=12)
8-12 weeks	↓	↓	↓	↓
	QA lesion to right striatum			
7 days	↓	↓	↓	↓
	Unilateral intrastriatal transplant of hCTX cell suspension ($5x10^5$cells)			
6 or 12 weeks post-transplant	↓	↓	↓	↓
	Animals sacrificed and brains cut for histological examination			

Table 5.5 Design of Experiment *iii)*, to validate the neonatal desensitisation method in the mouse using different cell numbers for neonatal injections. Hosts were desensitised between P0-2, received QA lesions from 8-10 weeks of age, and were unilaterally transplanted with hCTX 7 days post-lesion. Survival was assessed at either 6 or 12 weeks post-transplantation.

5.6.2 Results

Initial investigation of CV staining suggested some graft survival in all groups, with much healthier looking staining than observed in previous experiments (**Figure 5.8**, A-C, G-I). This was primarily observed in sections from animals which had been neonatally desensitised with the highest number of cells ($5x10^5$). However, this was again not reflected in HuNu staining, which showed one large graft, and little staining on other transplanted sections where only a few positive cells could be seen in contrast to the apparently large graft indicated by CV (**Figure 5.8**, D-F, J-L). Total numbers of surviving transplants are listed in **Table 5.6**.

Cells injected neonatally/treatment	Number of surviving grafts		Total (%)
	6 weeks	12 weeks	
$5x10^5$	0/6	1/5	9%
$1x10^5$	2/6	0/5	18%
$1x10^4$	0/3	1/3	16%
CsA	2/6	3/6	41%

Table 5.6 Number of surviving grafts in different transplant groups from Experiment *iii)* based on CV and HuNu staining.

Other methods of detection of transplanted human cells were unsuccessful as in Experiment *ii)*. Problems with high background staining were again experienced with this mouse tissue. A number of attempts were made to reduce this via the methods described in **Table 5.7**. The most successful method of accurate detection of transplanted human cells was found to be by using a biotin conjugation kit (Lightning Link, *Innova Biosciences*) to avoid the use of the anti-mouse secondary; reducing background staining (**Figure 5.9**). Despite these improvements to staining protocols, CV was still the main method of detection of surviving transplants, again suggesting overestimates may have been made in the number of surviving transplants, especially in the mouse graft groups. Surviving mouse transplants were small and pencil like, often with haemosiderin deposits. They could not be confirmed as surviving transplants and it is possible they show scarring and rejecting or rejected transplants with infiltration of host immune cells. No differences were observed in sections from hosts sacrificed at 6 or 12 weeks post transplantation (4/21 vs 5/19 across all treatment groups for each survival time).

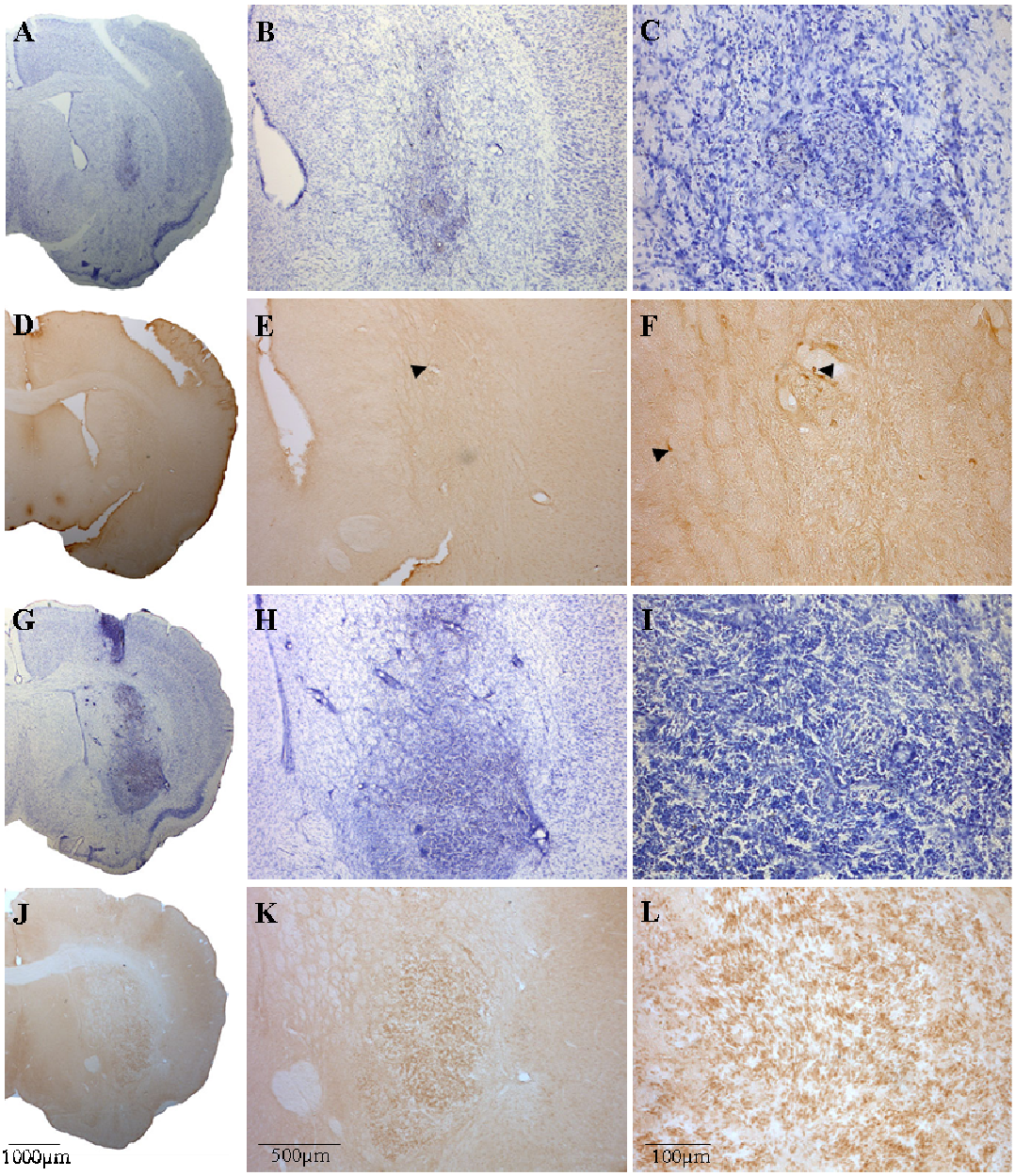


Figure 5.8 CV Staining in mouse hosts transplanted with hCTX after desensitisation with $5x10^5$ hCTX cells (A-C), or daily treatment with CsA 6 weeks after transplantation (G-I). CV staining appeared to show a number of surviving transplants however this was not confirmed with HuNu staining (D-F), where only a few positive cells could be seen (F-Arrowheads). G-L Shows a surviving transplant with clear positive HuNu staining.

Method		**Finding**
Alternative antibody	Human NCAM SC121 NF70	High background for all, still not possible to detect/quantify surviving human cells
Fluorescent secondary – May be clearer to identify positive cells than with DAB		High background, as above
Increase quench time to reduce binding to endogenous peroxidases		No noticeable improvement
Block with non-biotinylated anti-mouse secondary to bind non-specific binding sites		Some reduction in background but still not possible to detect/quantify
Antigen retrieval		No improvement
Biotin conjugate HuNu antibody (Lightning Link Biotin Conjugation Kit)		Reduced background and identification of surviving human cells (**Figure 5.9**)

Table 5.7 Methods tested to improve immunohistochemical staining of transplanted human cells in mouse tissue

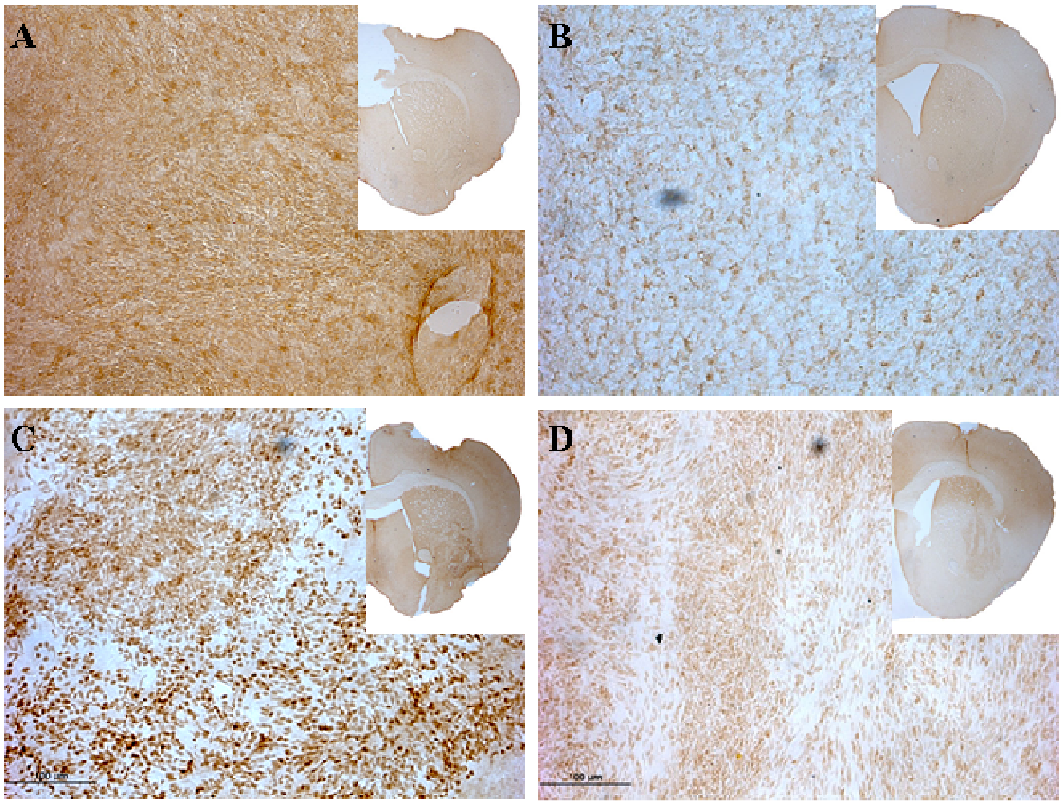


Figure 5.9 HuNu staining with biotinylated anti-mouse secondary (A, B) or with direct biotin conjugated primary and no secondary antibody (C, D) in two surviving human transplants in tolerised mice. Direct biotin conjugation reduced background staining and allowed clearer detection of human cells.

5.6.3 Discussion

For Experiment *iii)* protocols were modified to attempt to improve the survival of striatal xenografts. The number of cells neonatally injected was varied to determine the optimum number required to promote graft survival in this species. Other examples of the induction of neonatal tolerance in mice have used large numbers of spleen cells (e.g. $1.5x10^7 – 1x10^8$) injected neonatally (Ando *et al.* 1991; Peiguo *et al.* 2012), and studies suggest injecting larger cell numbers increases the potential of inducing tolerance (Peiguo *et al.* 2012). Successful induction of tolerance in neonatal hosts is thought to be dose-dependent, with the injection of low numbers of cells resulting in the opposite effect; priming of CTL (Adkins *et al.* 2004). This difference is thought to be due to the ratio between the number of injected donor cells and the number of circulating potentially responsive T cells within the host. To induce tolerance, all T cells which may be responsive must be switched off (Ridge *et al.* 1996). As successful neonatal desensitisation to neural transplants has to date been demonstrated using injections of $1x10^5$ human cells (Kelly *et al.* 2009b) in rats this is the number of cells which has been used as standard for the experiments in this thesis. It may be that injecting a higher or lower number of cells produces more consistent desensitisation and this could account for variability even in rat transplant experiments.

Additional modifications were made to the transplant protocol itself to attempt to improve graft survival. A higher number of cells were transplanted; $5x10^5$ as opposed to $3x10^5$, to improve the likelihood of survival of donor cells. Recent findings from our laboratory group have also suggested that grafting into the mouse striatum soon after the lesion (7-10 days) increases the chances of survival, agreeing with reports from others suggesting that a shorter time between lesion and transplant may be beneficial (Johann *et al.* 2007). In this study, allogeneic NSC transplants were delivered to the QA lesioned mouse striatum 2, 7 and 14 days after the lesion. Although looking at relatively small animal numbers, the authors reported some astrogliosis and microglial activation 2 days after the lesion which was significantly elevated at 7-14 days. Larger surviving grafts were found in early transplants which were attributed to this, suggesting that a change in morphology of microglia may indicate a switch from a neuroprotective to neurotoxic role. Earlier grafting time-

points were therefore used in this experiment as an additional attempt to improve graft survival by avoiding potential neurotoxic effects of activated microglia following the lesion.

On initial investigation of CV stained sections from this experiment, it was thought that a number of transplants had survived and primarily in hosts which had been injected neonatally with the highest number of cells ($5x10^5$). This would be in line with previous research into neonatal tolerance, demonstrating the induction of tolerance to allogeneic skin grafts in adulthood following a neonatal injection of a suspension of a high number of cells (between ~$1.5x10^6$ and 10^7) (Adkins *et al.* 2004; Modigliani *et al.* 1997; Ridge *et al.* 1996; West *et al.* 1994). Based on this CV staining, which was suggestive of good transplant survival, Experiment *iv)* was started, in which the number of cells injected neonatally was increased further. However HuNu staining from Experiment *iii)* was subsequently found to be minimal, as in previous experiments. It was unclear whether this was due to problems with immunohistochemical staining protocols or due to a lack of surviving human cells, since CV staining was convincing as to the presence of surviving grafts. In order to identify why this may be the case; antibodies for a panel of immune markers were tested. Sections from this experiment, as well as from a pilot experiment carried out with BL/6 mouse hosts were used to evaluate these stains. Details for this pilot experiment can be found in **Appendix 7**, in which BL/6 mouse hosts were desensitised with human tissue to determine whether successful transplant survival could be promoted in this strain of mouse. Although survival was again poor, this allowed the detection of an intense infiltration of microglia in the transplanted area corresponding to the CV staining. This highlights a potential problem with the use of CV staining in the detection of transplanted cells where there is no specific antibody or cell label available, and suggests that a lack of HuNu+ cells in these “graft” areas is due to an elevated inflammatory response.

5.7 Experiment *iv)* Striatal xenografts in mouse hosts desensitised with hPF cell numbers increased above Experiment *iii)*

5.7.1 Experimental Design

Four litters of mice (n=40; 21F, 19M) were injected neonatally with a suspension of differing numbers of cells from hCTX tissue (**Table 5.8**). Hosts either received QA lesions to the right striatum in adulthood (n=20), or the tissue was left intact (n=20). This was followed by a transplant either 2 or 8 days later of hCTX tissue ($5x10^5$ cells) as in previous experiments. All groups were split equally between desensitisation, lesion and transplant conditions. Survival was then assessed 12 weeks after transplantation.

P0-2	hCTX tissue ($5x10^5$ cells) injected i.p. (n=11)	hCTX tissue ($1x10^6$ cells) injected i.p. (n=11)	hCTX tissue ($2.5x10^6$ cells) injected i.p. (n=10)	hCTX tissue ($5x10^6$ cells) injected i.p. (n=11)
6-12 weeks	↓	↓	↓	↓
	QA lesion to right striatum or no lesion			
2-8 days	↓	↓	↓	↓
12 weeks post-transplant	Unilateral intrastriatal transplant of hCTX cell suspension ($5x10^5$ cells)			
	↓	↓	↓	↓
	Animals sacrificed and brains cut for histological examination			

Table 5.8 Design of Experiment *iv)* Desensitisation of mouse hosts with large numbers of hCTX cells

5.7.2 Results

Sections were stained with CV to identify surviving grafts. Subsequent staining with HuNu (biotin conjugated) revealed few surviving grafts overall (10%) despite additional modifications to the desensitisation and transplantation protocols. Numbers of surviving grafts are displayed in **Table 5.9**. The greatest number of surviving grafts were seen in animals desensitised with the lowest number of cells ($5x10^5$), however as there were still only 2 out of a total of 10 transplanted this cannot be considered to be conclusive. **Figure 5.10** shows the largest surviving graft. Some sections from other animals showed staining with CV; however no human cells could be detected with immunohistochemical staining for HuNu. These are displayed in **Figure 5.11** in comparison to the positive staining found in the largest transplant.

Cell number injected neonatally	**Lesion**	**Number of surviving grafts**	**Total %**
$5x10^6$	QA	1/5	10%
	None	0/5	
$2.5x10^6$	QA	1/4	11%
	None	0/5	
$1x10^6$	QA	0/6	0%
	None	0/5	
$5x10^5$	QA	1/5	20%
	None	1/5	
		Total	10%

Table 5.9 Numbers of surviving xenografts in desensitised mice from Experiment *iv)*

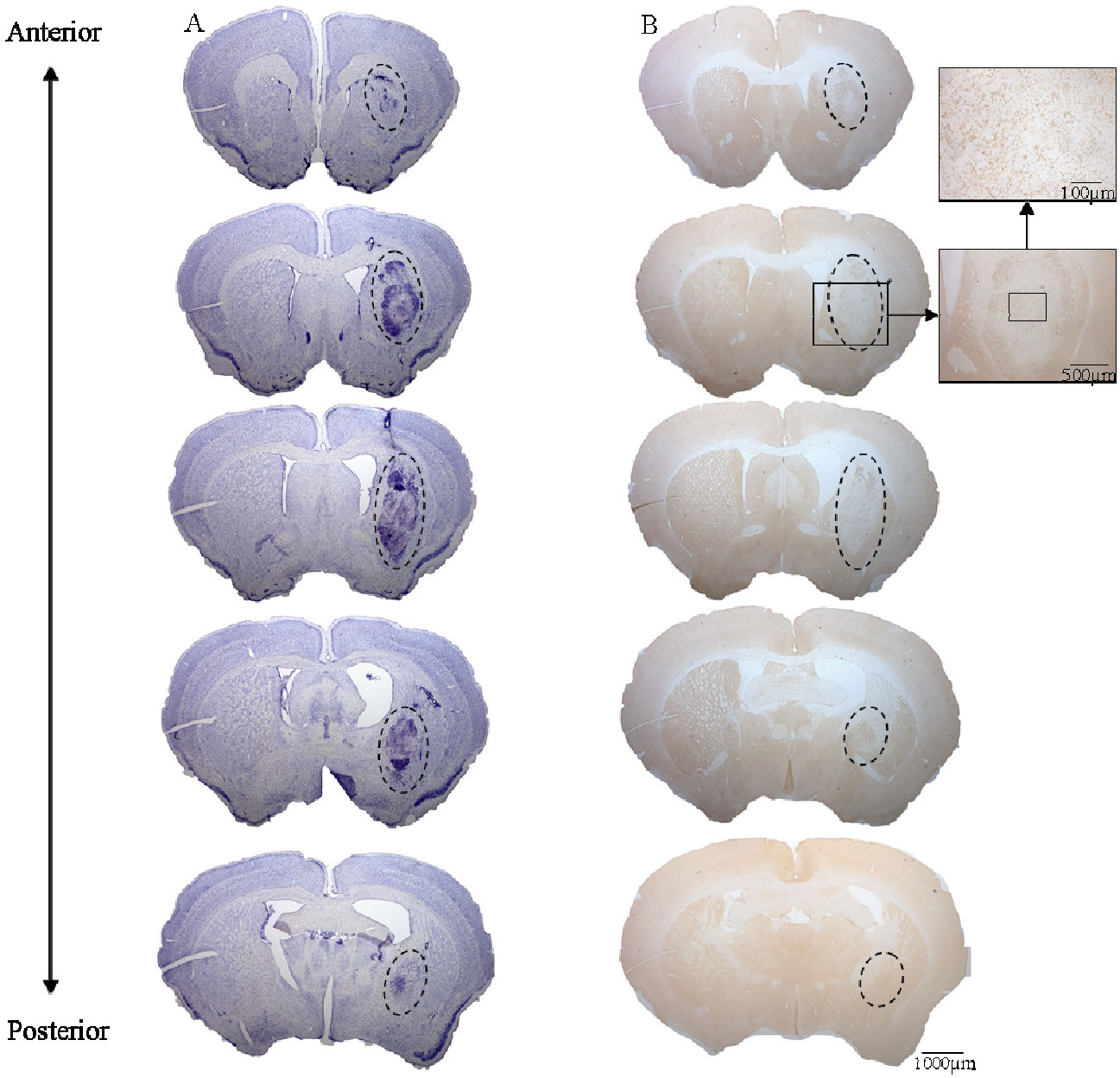


Figure 5.10 Surviving human graft 12 weeks after transplantation in a mouse tolerised with $5x10^5$ human foetal neural cells. A) shows a series of sections stained with CV with the grafted area outline. B) shows comparable sections stained with HuNu, showing surviving transplanted human cells. Few surviving transplants were found but were often large like this one, spanning the whole striatum

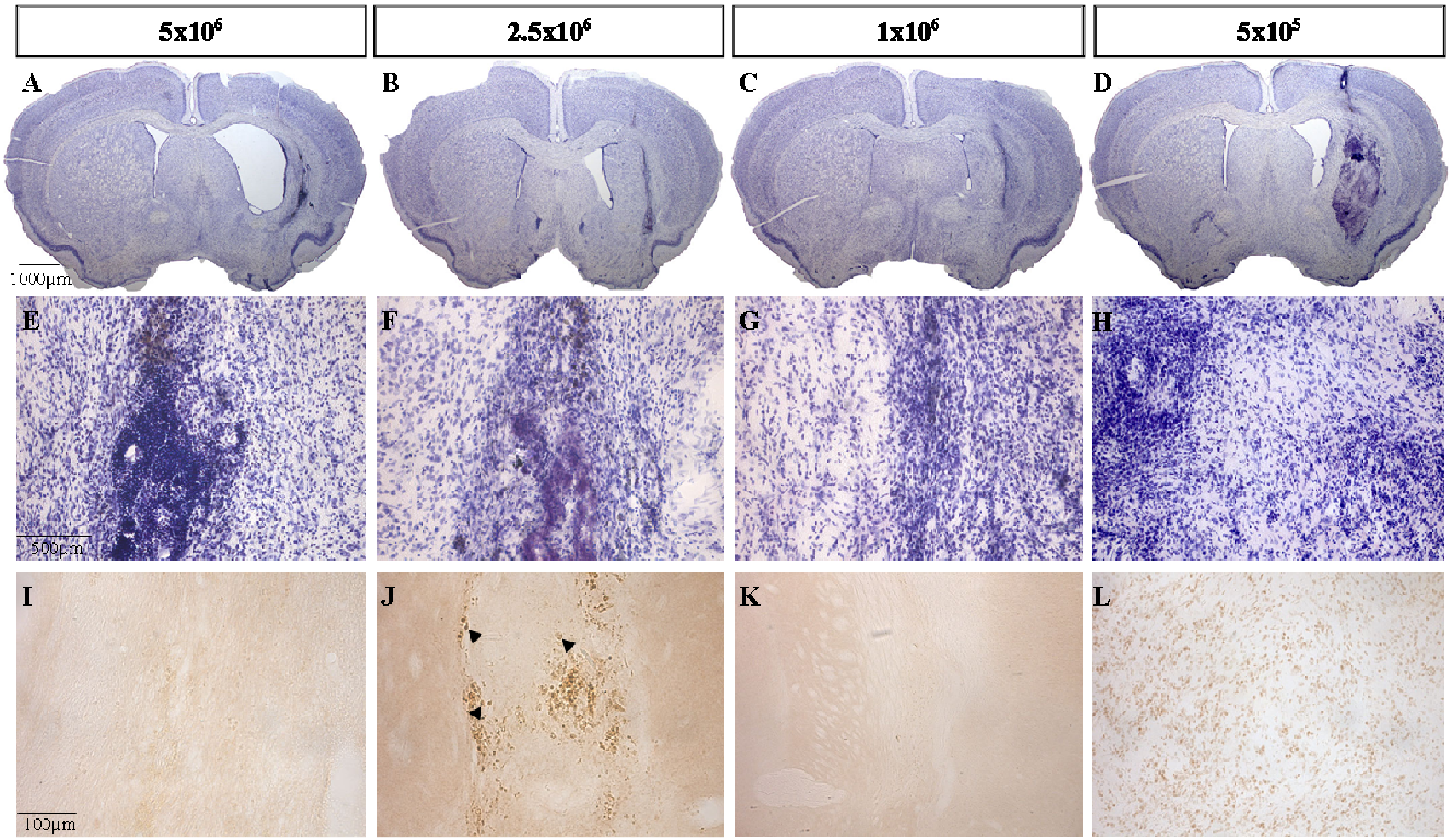


Figure 5.11 Sections from mice tolerised with $5x10^6$ (A,E,I), $2.5x10^6$ (B,F,J), $1x10^6$ (C,G,K) and $5x10^5$ hCTX cells (D,H,L) stained with CV (A-H) and HuNu (I-L). Positive HuNu Staining can be seen in L, other sections show either no staining, or dead cells/scarring as in J despite the appearance of surviving transplants on CV stained sections

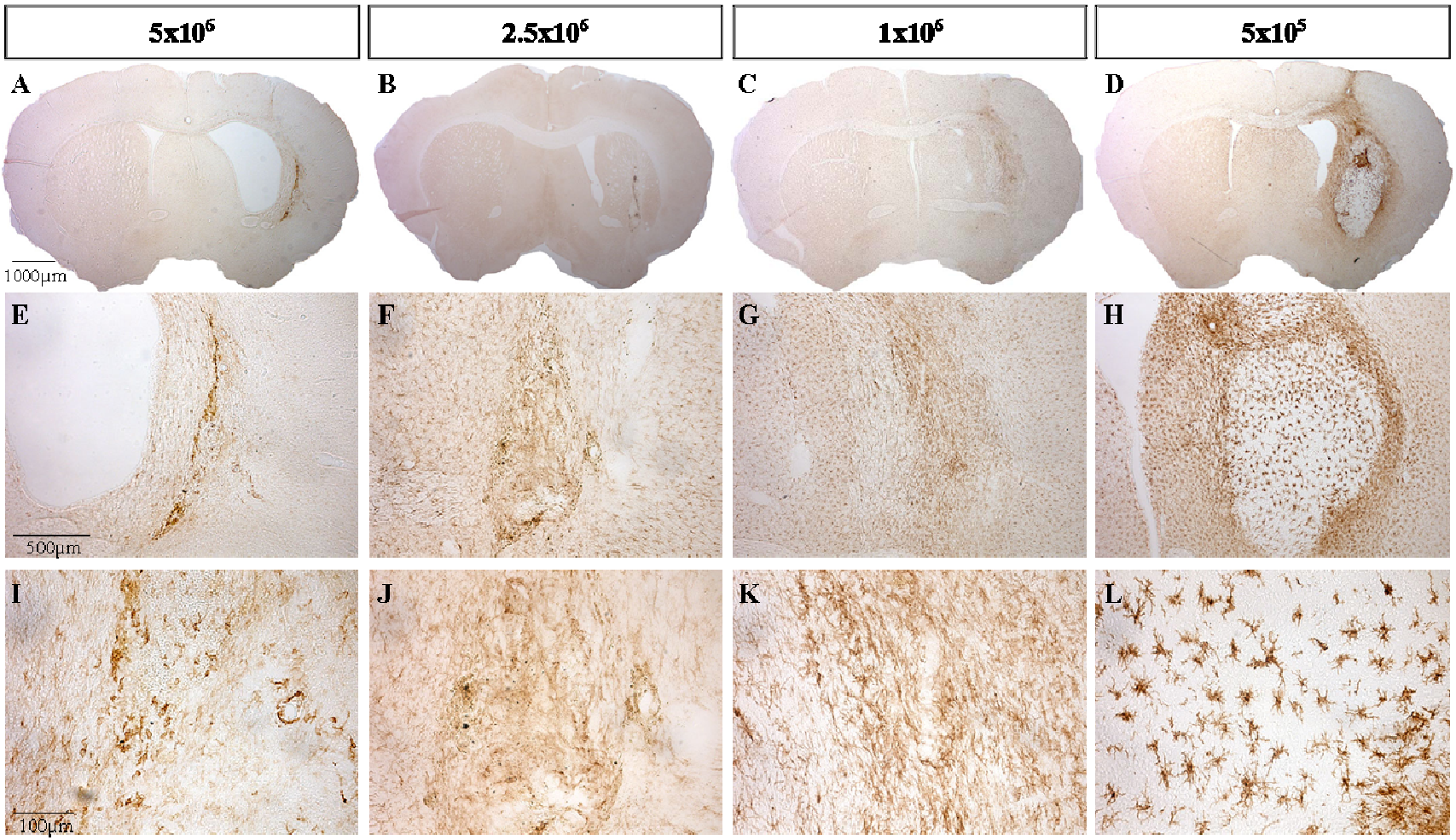


Figure 5.12 Iba1 staining of microglia in the grafted area in desensitised mice. Staining shows microglial infiltration into the grafted area in sections where no surviving human cells were identified (A-C, E-G, I-K) with an activated appearance (I). Staining was still increased around the area of a surviving transplant, however the majority of cells resembled ramified resting microglia (D, H, I)

To study the immune response to neural xenografts, a 1:12 series of sections was stained for the microglial marker Iba1. The extent of immunoreactivity was graded according to the rating scale used previously (Duan *et al.* 1995; Larsson *et al.* 1999). This is presented in **Figure 5.13**, showing a strong microglial response to hCTX transplants in desensitised hosts. In particular, in the case of rejected grafts dense infiltration of activated microglia was observed correlating with the graft area as shown in CV stained sections (**Figure 5.12** A-C, E-G, I-K) as compared to the ramified microglia observed surrounding the surviving transplant in **Figure 5.12** (D,H,L).

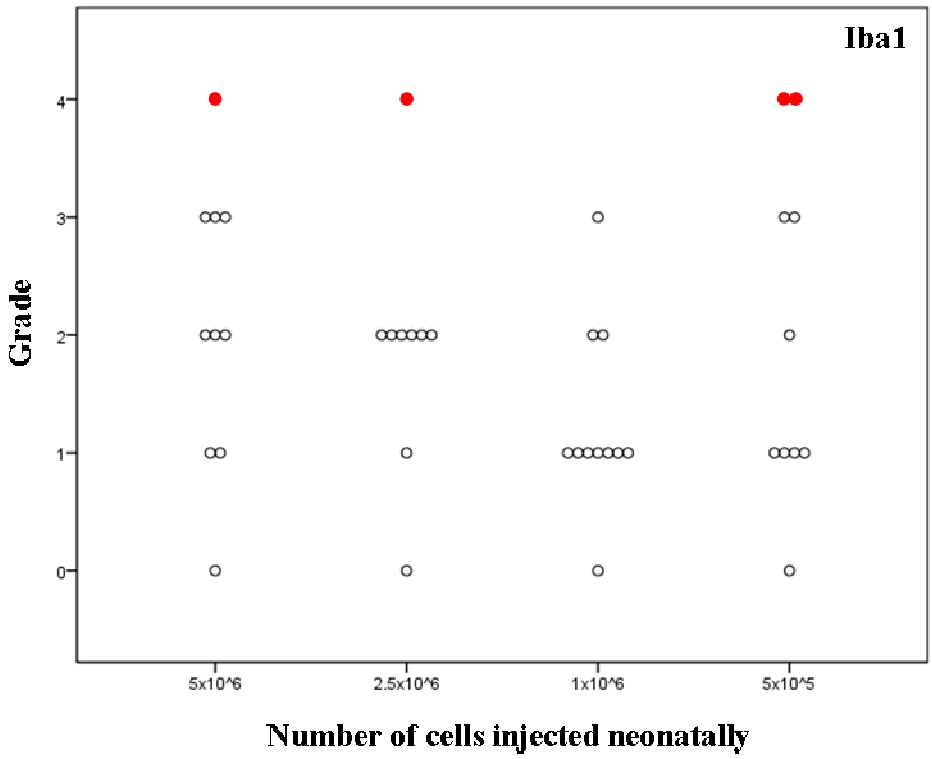


Figure 5.13 Iba1 immunoreactivity showing microglial activity around the grafted area in mouse hosts transplanted with hCTX following desensitisation with varying numbers of cells. Each circle corresponds to one host animal, with red circles showing surviving transplants

5.7.3 Discussion

Mouse hosts included in this experiment were desensitised neonatally with large numbers of hCTX cells in order to attempt to promote the survival of human xenografts subsequently transplanted to the adult striatum. This experiment was started during the assessment of data from Experiment *iii)*, when it was thought that desensitisation using a higher number of cells was successful. In line with previous data, showing that very large numbers of allogeneic cells injected neonatally induced tolerance to transplants of tissue from the same strain of mouse (Adkins *et al.* 2004; Modigliani *et al.* 1997; Ridge *et al.* 1996; West *et al.* 1994), up to $5x10^6$ hCTX cells were injected neonatally. Experiment *iii)* attempted to improve graft survival by transplanting 10 days after the lesion due to previous data from this group suggesting that this timepoint improved survival into mouse hosts (unpublished data). A recent study tested the optimum time for delivery of cell transplants into the QA lesioned mouse striatum with stem cells transplanted at 2, 7 and 14 days post-lesion (Johann *et al.* 2007). The authors found that the best survival was observed in transplants delivered 2 days after the lesion, and suggest this to be due to the presence of only moderate host astrocytic and microglial activation at this time-point as compared to the others. Therefore, in this experiment equal numbers of hosts either received no lesions, or were transplanted only 2 or 8 days post-lesion.

Assessment of graft survival, however, showed very few surviving grafts (10%) overall. Data suggests that, in fact, the lowest number of cells used for neonatal desensitisation ($5x10^5$) promoted the best survival. However since there were still only 2 surviving grafts identified in this group it is more likely that desensitisation was not successful overall. With regards to the lesion conditions; again since survival was so low it was impossible to attribute any survival differences to the time transplants were delivered after the lesion, or indeed whether animals received QA lesions at all.

The host immune response was studied through subjective quantification of microglial staining. A dense infiltration of activated microglia was observed in a number of transplants, the majority of which had not survived. This finding was consistent with the staining observed in a study of hCTX transplant survival in desensitised BL/6 hosts (**Appendix 7**). Together these findings suggest an effect of

the inflammatory response in rejection of transplants to the mouse striatum, which agrees with the findings from Experiments *i-iii)*, in which CsA treatment did not improve graft survival.

5.8 Combined Results

The results of each experiment for this chapter have been reported individually showing small numbers of surviving transplants in all experiments. Here the data has been compiled together from groups testing the same transplant conditions in order to identify any trends. **Table 5.10** shows all of these data grouped by treatment and lesion/transplant condition. Additionally, total numbers of surviving transplants are calculated for both mouse grafts (7/18) and xenografts (27/137). Graft survival is grouped together irrespective of the time between lesion and transplant, and the time post-transplant at which survival was assessed. These data highlights the poor levels of survival in transplanted mice, despite the expectation that mouse tissue transplant groups, desensitised groups, and CsA treated groups may all tolerate surviving transplants.

5.8.1 Effect of treatment type on transplant survival

Comparing graft survival following treatment with CsA, neonatal desensitisation with hCTX (all cell numbers), and untreated hosts showed no difference in survival between desensitised animals and those receiving no treatment (16% and 15% respectively; **Figure 5.14**). Mice treated with CsA showed the highest rate of xenograft survival (46%) as compared to desensitised or untreated hosts. The effect of CsA treatment on both mouse graft and human xenograft survival is compared in **Figure 5.15**. Treatment with CsA did improve survival of xenografts as compared to untreated hosts, however only increasing survival up to 48%, as compared to 18% in untreated hosts. However, hosts receiving mouse tissue transplants which were treated with CsA daily had lower numbers of surviving grafts than those which were untreated (12.5% vs 54.5%).

5.8.2 Desensitisation with varying cell numbers

Desensitised hosts in Experiments *iii)* and *iv)* received neonatal injections of varying numbers of hCTX cells (between $1x10^4$ and $5x10^6$) followed by transplants in adulthood of hCTX tissue ($5x10^6$ cells). Low survival was seen in all transplant groups, therefore data were not statistically analysed. **Figure 5.16** shows the data from these experiments, suggesting that graft survival is optimal using around $1x10^4$

and $1x10^5$ cells and poorer with the use of higher cell numbers to desensitise, although these are only based on very low numbers of surviving transplants.

5.8.3 Transplants to the lesioned or intact striatum

A range of different lesion and transplant designs were used in the experiments in this chapter. Some transplants were delivered to the intact striatum, whereas the majority were delivered into QA lesioned hosts. Again, although surviving transplant numbers were not high enough to elucidate whether this had a statistically significant effect on graft survival, data compiled from all experiments and represented in **Figure 5.17** suggests that lower numbers of grafts survived when transplanted to the intact striatum. However; as discussed in Experiment *i),* since CV staining in lesion only animals was often observed to be comparable to that found in transplanted hosts and positive identification of surviving transplants was not obvious, firm conclusions cannot be drawn.

Treatment group	Number of cells for desensitisation	Lesion type	Transplanted tissue type	Number of cells transplanted	Number of grafts surviving (% of grafts surviving)
DhCTX	1×10^4	QA	hCTX	5×10^5	**1/6 (17%)**
DhCTX	1×10^5	QA	hCTX	5×10^5	**2/11 (18%)**
DhCTX	5×10^5	QA	hCTX	5×10^5	**1/16 (6%)**
DhCTX	5×10^5	-	hCTX	5×10^5	**1/5 (20%)**
DhCTX	1×10^6	QA	hCTX	5×10^5	**0/6 (0%)**
DhCTX	1×10^6	-	hCTX	5×10^5	**0/5 (0%)**
DhCTX	2.5×10^6	QA	hCTX	5×10^5	**1/4 (25%)**
DhCTX	2.5×10^6	-	hCTX	5×10^5	**0/5 (0%)**
DhCTX	5×10^6	QA	hCTX	5×10^5	**1/5 (20%)**
DhCTX	5×10^6	-	hCTX	5×10^5	**0/5 (0%)**
DhCTX	1×10^5	QA	hCTX	3×10^5	**5/27 (19%)**
DhCTX	1×10^5	-	hCTX	3×10^5	**1/5 (20%)**
-	-	QA	hCTX	3×10^5	**2/13 (15%)**
CsA	-	QA	hCTX	3×10^5	**7/13 (54%)**
CsA	-	QA	hCTX	5×10^5	**5/12 (42%)**
-	-	QA	mWGE	3×10^5	**6/11 (55%)**
CsA	-	QA	mWGE	3×10^5	**1/8 (12.5%)**
				Total hCTX survival	**27/137 (20%)**
				Total mWGE survival	**7/18 (39%)**

Table 5.10 Data compiled from Chapter 4, showing number of surviving transplants in each group and total number of surviving transplants across groups. hCTX = human embryonic cortex, mWGE = mouse embryonic whole ganglionic eminence, QA = quinolinic acid lesion, DhCTX = desensitisation with hCTX cell suspension, CsA = immunosuppression with daily injection of cyclosporine A. (Reproduced from Roberton et al 2013)

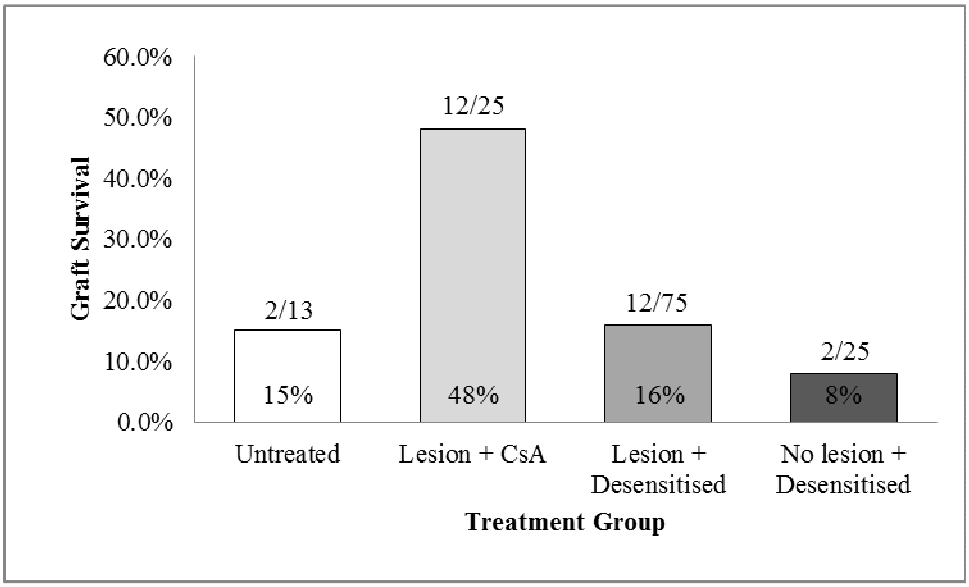


Figure 5.14 Comparison of treatment groups in mice receiving xenografts of hCTX to the striatum. *Survival percentages are displayed at the base of bars, with the number of hosts in each group at the top.*

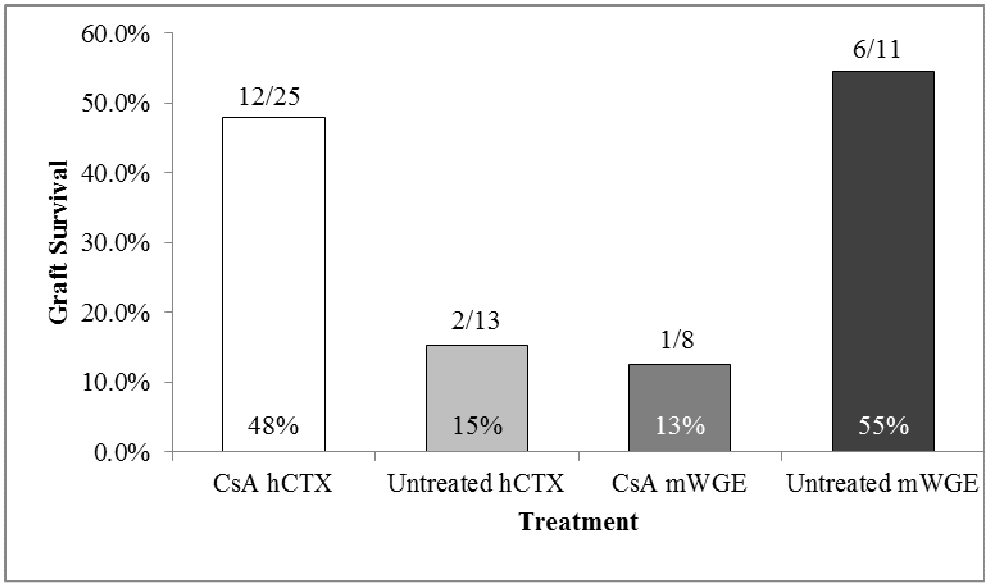


Figure 5.15 Comparison of CsA immunosuppression in mWGE and hCTX transplant groups. *Survival percentages are displayed at the base of bars, with the number of hosts in each group at the top.*

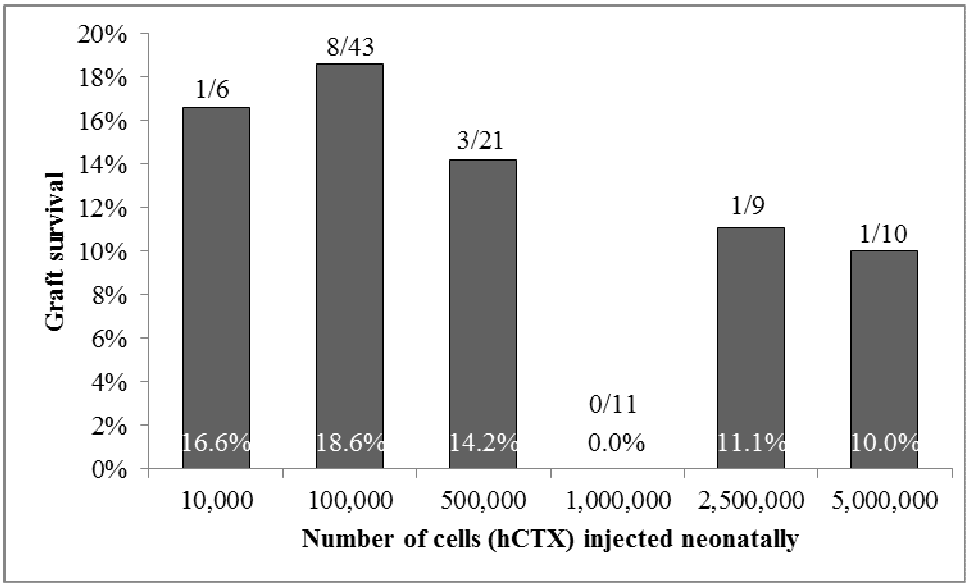


Figure 5.16 Comparison of graft survival in mouse hosts desensitised with a range of cell numbers. *Survival percentages are displayed at the base of bars, with total numbers shown at the top.*

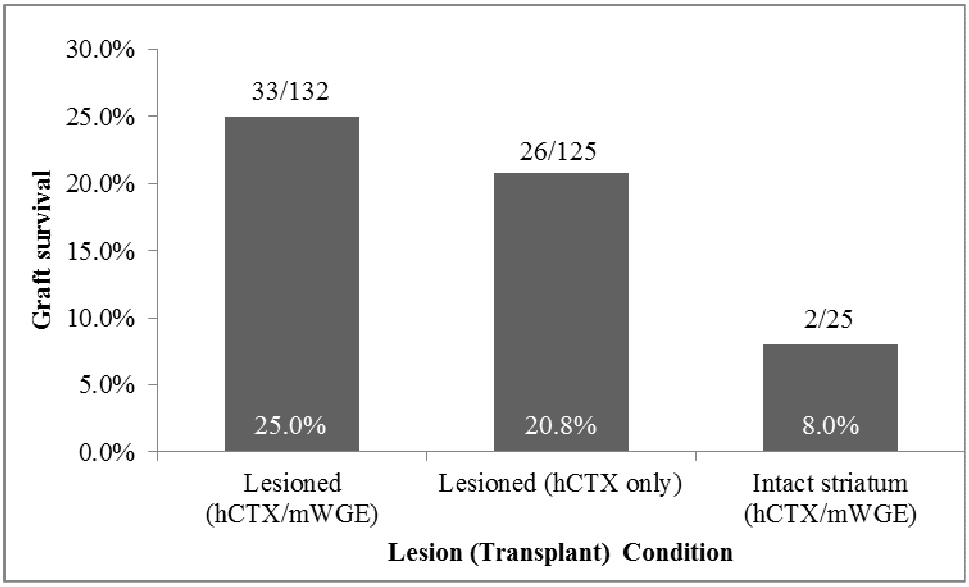


Figure 5.17 Survival of transplants of allogeneic and xenogeneic tissue into the lesioned and unlesioned mouse striatum. *Survival percentages are displayed at the base of bars, with total numbers shown at the top.*

5.9 Chapter Discussion

The aim of this chapter was to validate the neonatal desensitisation method for use in mouse hosts. It was hypothesised that mice injected (i.p) neonatally with cell suspensions of hPF tissue would show greater survival rates of subsequent neural transplants of the same tissue type than those mice which were untreated. The combined results from these experiments do not show successful desensitisation of CD-1 mouse hosts, and highlight a problem with poor survival of both mouse grafts and human xenografts in the mouse striatum.

5.9.1 Desensitisation in mouse hosts

Experiment *i)* aimed to test the survival of human xenografts in hosts which had either been desensitised to human tissue in the neonatal period or untreated. A mouse transplant group was included to confirm the success of the transplantation protocol itself, however due to the initial assumption that xenograft survival in immune suppressed hosts was consistently successful; a CsA treated group was not included in this experiment. Data showed poor graft survival in desensitised hosts, with positive staining of human cells hard to identify. Due to the absence of a CsA treated control group, it was not possible to confirm whether this poor graft survival could be attributed to the unsuccessful desensitisation of hosts, or to problems with donor tissue or other aspects of the xenograft procedure. There was also poor survival of mouse transplants, potentially indicating a problem with the transplant procedure more generally.

In order to address these problems, Experiment *ii)* included an immune suppressed control group, receiving daily injections of CsA (10mg/kg) to inhibit the T cell response to transplanted cells. A group of hosts in this experiment also received transplants of mouse donor tissue from the same strain, half of which were treated with CsA to try and promote survival. Again, poor survival was found in all groups. Survival of mouse grafts was not improved by CsA treatment, and in fact appeared worse than in untreated hosts. CsA treatment also did not promote survival in xenografted hosts. A poor effect of CsA as an immunosuppressant in neural transplantation has been reported previously (Jablonska *et al.* 2013; Larsson *et al.* 2001b; Walczak *et al.* 2004), however in our hands the use of CsA promotes fairly

consistent transplant survival in rat hosts. Since transplant survival was poor in all groups, including those receiving allogeneic tissue, a number of modifications were made to attempt to improve transplant survival. This included adjusting the number of cells transplanted and the time between lesion and transplant, as discussed below.

Experiments *iii)* and *iv)* tested the use of increased number of cells for desensitisation of neonatal mouse hosts. As discussed this was based on previous literature on neonatal desensitisation in mice to peripheral transplants of allogeneic tissue. Despite success using this method, it has not yet been successful using xenogeneic tissue. This could be due to species specific differences between donor and host tissue, for example due to the presentation of antigen on donor cells by foreign MHC which may not be recognised by the host. By taking advantage of a transgenic mouse model expressing the human MHC molecule HLA-B7 it has previously been demonstrated that specific tolerance could be induced to HLA-B7. This suggests the structure of xenogeneic donor MHC molecules may not be the preventative factor in the reliable induction of neonatal tolerance to xenogeneic tissue (Borenstein *et al.* 2004). In assessing the levels of chimerism through staining of host spleen cells for HLA-B7, the authors found HLA-B7^{+} cells in peripheral lymphoid organs (albeit at low levels) suggesting engraftment of donor cells into recipient bone marrow. This implies a correlation between chimerism and tolerance. In our model it is possible that this induction of chimerism has not been achieved and therefore the induction of tolerance is unsuccessful.

Following successful induction in mice of tolerance to allogeneic tissue following i.v. injection of donor splenocytes in adulthood, Wang *et al* attempted to induce tolerance to islet xenografts (rat) using the same method. Prolonged survival of xenotransplants was found, however tolerance could not be achieved without transient B cell depletion with a CD20 antibody. Following this depletion, a later challenge showed the hosts were no longer capable of generation of anti-rat antibodies (Wang *et al.* 2013b). However as these studies were carried out in adult hosts it is not clear whether the same would apply in this case of neonatal tolerance to xenogeneic tissue.

5.9.2 Modification to transplant protocols

In order to improve the survival of transplants into mouse hosts, a number of modifications were made to the transplant protocol, as well as changing the numbers of cells used to desensitise neonatally. In initial mouse experiments; hosts received a transplant of $3x10^5$ cells between 1-2 months after a QA lesion. In attempts to improve survival the number of cells transplanted was increased to $5x10^5$ cells; the number of cells used as standard to transplant into rat hosts. However, no improvement was found in transplant survival in those animals transplanted with higher cell numbers. It is possible that instead of increasing the chances of some cells surviving, the number of cells was too high. The lesioned striatum of the mouse host is smaller than that of the rat and may not be able to support a deposit of such a large cell number, actually increasing the chance of rejection.

In addition to modifying the number of cells transplanted, the time between lesion and transplant was altered in a number of transplant groups in this chapter. Döbrössy *et al* found good transplant survival of mouse tissue implanted 10 days after a lesion (Döbrössy *et al.* 2011), which has been replicated in this lab group (Harrison D.J., *unpublished data*) and Johann *et al* compared the survival of cells transplanted at 2, 7, and 14 days post-lesion and found the greatest survival in their earliest transplant time-point (Johann *et al.* 2007). In the studies presented here, no improvements were observed in transplants delivered at shorter time-points following the lesion, even up to 2 days. It appears that survival of transplants in lesioned hosts is better than those implanted into the intact striatum; however this analysis was complicated by problems with the identification of transplanted cells, as discussed below.

Another consideration was the transplant co-ordinates used. In all studies transplant co-ordinates used were the same as those used for QA lesions. In contrast to rat lesions which are delivered to two sites in the striatum, in the mouse only one site is used. Therefore, whereas in the rat transplant scar tissue from the previous surgery is avoided, the mouse transplant is delivered to the same site due to the small target area. Therefore it is possible the transplant is deposited into a more hostile environment.

5.9.3 Identification of grafted cells

Difficulties in the positive identification of transplanted cells were experienced in all experiments in this chapter. Initial identification of surviving grafts in all cases was performed using CV staining to determine the presence of a dense deposit of cells within the striatum. In transplants into the rat striatum, large transplants can clearly be identified in this way (see **Chapters 3** and **4**). In transplants into mouse hosts this is less clear, with smaller surviving grafts frequently observed (El-Akabawy *et al.* 2012; Johann *et al.* 2007; Kelly *et al.* 2007), more resembling a thin pencil shaped graft or a needle track/scar of a rejected transplant. In the case of transplants of mouse cells, which have not been labelled prior to transplantation, clear identification is only possible in transplants into the QA lesioned striatum where a clear margin of lesion can be seen beyond all graft borders. In this case a clear deposit of transplanted cells can be identified with antibody staining for NeuN-positive neurons and labelling with DARPP-32 to identify the presence of mature MSNs. Sections containing mouse tissue transplants from these experiments were damaged prior to staining with these markers, making it impossible to confirm the presence of surviving transplanted cells (See **Appendix 5**).

Although with xenografts of human cells a number of human specific antibodies are available, problems were experienced with the use of these in identification of surviving transplants. Experiments *i)* and *ii)* found poor antibody staining, potentially due to underfixing of tissue sections. Additionally the majority of these human specific antibodies are raised in mouse, requiring the use of a biotinylated anti-mouse secondary antibody to visualise positively stained cells with DAB. Due to the nature of the experiments, damage has been sustained to BBB as a result of surgery for lesions or transplants. Although this repairs after a variable period of time, dependent on the inflammatory response to lesions and transplants (Sanberg *et al.* 1988; Wakai *et al.* 1986), it is likely that immunoglobulins will have passed through the BBB and be present in the brain, specifically in the transplanted area. This causes high background staining from the anti-mouse secondary antibody, making it difficult to identify positive staining. Due to these difficulties in labelling, numbers of surviving grafts recorded may be overestimates identified from examination of CV stained sections.

Although some improvement was found with the use of a biotin conjugation kit, allowing avoidance of the use of the anti-mouse secondary, positive staining of transplanted human cells still did not correlate with CV staining. Staining in Experiment *ii)* of lesion only control animals showed comparable staining to that observed in a number of grafted animals, and indeed to that previously considered to be indicative of surviving mouse grafts. In light of this, when human cells could not be positively identified despite the appearance of a surviving graft in CV stained sections, attempts were made to determine whether this staining was due to the presence of immune cells in the transplanted area. This was carried out on sections from desensitised BL/6 hosts transplanted with hCTX (**Appendix 7)**. Staining showed a lack of NeuN positive neurons in the CV stained area, but dense microglial staining. This suggests the transplant has been infiltrated by host microglia and is undergoing rejection.

5.9.4 Graft survival in mouse hosts

It is not clear why graft survival in mouse hosts is so poor in comparison to the rat. Even in hosts receiving mouse tissue transplants in these experiments, although survival was higher than xenograft groups, it still only reached ~50%. There are a number of possibilities as to why this may be the case which are currently undergoing investigation. It may be due to the size of the mouse brain, i.e. it cannot support a transplant of this number of cells, and even $3x10^6$ cells is too many to produce a healthy surviving transplant. Alternatively, there may be something innately different about the mouse immune system. A recent paper highlights the role of the innate immune response in the rejection of cells transplanted into the mouse brain (Phillips *et al.* 2013). It has been assumed that since donor cells used in neural transplantation generally express low levels of MHC-I and II, they are relatively protected from rejection by the immune system. However this may not be the case, since up-regulation of MHC molecules occurs in response to pro-inflammatory cytokines upon transplantation, moreover this may not necessarily be beneficial. Phillips *et al* confirmed this, finding that the responses from NK cells in mouse hosts is elevated on encountering cells with low MHC expression (Phillips *et al.* 2013).

Data from these experiments suggests a strong microglial response to xenotransplants of hPF tissue in the mouse striatum (Experiments *iii)* and *iv)*). Although this is suggestive of an increased inflammatory response, staining for T cells ($CD4^+$ and $CD8^+$) did not show a large number of lymphocytes in or around the grafted area. Aside from presenting antigen to T lymphocytes, it is possible for microglia themselves to act as cytotoxic effector cells or innate phagocytes (Armstrong *et al.* 2001; Davis *et al.* 1994; Fanger *et al.* 1989). The administration of CsA daily did not improve survival of transplants greater than 50%, or appear to reduce the amount of microglial staining in and around the grafted area. This agrees with a previous study in which the administration of transient or continuous CsA to promote survival of hNSCs in an ischemic rat model was not found to reduce the inflammatory response as measured by Iba1 staining, even in animals which only received lesions (Rota Nodari *et al.* 2010). Investigating the phenotype of the microglia present at the transplantation site will give insight into this phenomenon and allow further understanding of the rejection response in the mouse and targeting of relevant immune suppression treatments. Additionally, it has been reported that high doses of CsA are required for successful immunosuppression in mice, although this is difficult to achieve successfully due to side effects (Larsson *et al.* 2001b). The problems of transplant survival observed here do not appear to be isolated to findings in this lab group, with the majority of other centres using immune compromised hosts to successfully carry out transplants of human donor cells, presumably due to issues with rejection in wild type mouse hosts (De Filippis *et al.* 2007; Espuny-Camacho *et al.* 2013; Liang *et al.* 2013; Ma *et al.* 2012; Wang *et al.* 2013a).

A recent paper aimed to determine whether mouse hosts could be desensitised neonatally to a range of cell types including hiPSCs and hNPCs (Mattis *et al.* 2014). The authors did not find any survival of human grafts in their desensitised hosts beyond 2 weeks post-transplantation. These experiments also investigated the survival of transplants of human iPSC derived NPCs (iPSC-NPCs) and foetal NPCs (hfNPCs) in neonatal mouse hosts. Again, rejection of human donor cells was observed, as compared to transplants into NOD/SCID mice which survived up to 62 days after transplantation. The authors also conclude the survival of transplants in mice to be poorer to rat, and attribute unsuccessful neonatal desensitisation to this.

Moreover the authors note a large host microglial response to the human xenografts (Mattis *et al.* 2014), as found in the studies presented here.

A further consideration from these studies is the characteristics of surviving human transplants. Although very few in number, the majority of transplants which did survive were very large. This could result from the preparation of cell suspensions for transplantation, in which tissue may not have been adequately dissociated into a single cell suspension and may instead contain small portions of tissue. In this scenario; when transplanting each host animal from one cell suspension, one or more may receive transplants of these tissue portions whereas others may receive very few cells at all. The delivery of pieces of tissue may mean that one host animal receives the majority of cells from the cell suspension, or that the cells have a better chance of surviving when delivered in this way. Although previous work has looked into the effect of transplantation of tissue pieces as opposed to single cell suspensions in rat hosts, this has not been performed in mice. These issues are addressed in **Chapter 6**, in which the preparation of mouse donor tissue for transplantation into the mouse brain is investigated to determine whether delivering cells as tissue pieces has an effect on graft survival.

Similarly large grafts were seen in human to rat transplants in previous studies presented in this thesis which were found to be due to extensive proliferation of developing cortical tissue, thus this provides an alternative explanation for the marked variability in hCTX graft sizes in mouse hosts. As large numbers of cells were transplanted in mouse hosts (up to $5x10^5$) to try and improve survival, further proliferation of this tissue would result in large grafts. If the survival of transplants could be improved this number may be reduced to produce slightly smaller transplants. This also may not be an issue with the use of striatal tissue transplants from WGE.

5.10 Conclusions and future work

The objective of the studies described in this chapter was to determine whether mouse hosts could be successfully desensitised in the neonatal period to human cortical tissue to a sufficient extent to promote survival of a neural xenograft in adulthood from 6-12 weeks post-transplantation. Successful desensitisation could not be demonstrated. In addition to this, poor graft survival was found in the majority of mouse hosts. This has identified a potential problem with the use of mouse hosts for testing donor cells for transplantation in neurodegenerative disease. Ideally transplants could be delivered to lesion or genetic models of disease in order to measure safety and efficacy of donor cells. However, data from this chapter suggests that the immune system of the mouse host may not provide an ideal environment for cell transplantation. **Chapter 6** aims to address problems identified with mouse tissue transplant survival, before research can be redirected to identify whether neonatal desensitisation to xenogeneic tissue in the mouse may be a possibility.

In addition to the mouse to mouse transplant experiment described in **Chapter 6**, future studies will look to improve survival of human xenografts in mouse hosts. Specifically; a systematic investigation into the mouse (CD-1) immunological response to striatal transplants of hCTX will be carried out, with a direct comparison to the response in the rat (SD). The response to transplants will be investigated in both untreated and immune suppressed hosts treated daily with CsA, with an investigation of the T cell and microglial response to transplants at various time-points following transplantation. This study will aim to identify differences in the response of mouse and rat hosts to transplants of human tissue, and therefore allow immune suppression treatments to be more specifically targeted to the relevant rejection processes. An additional investigation of survival of human xenografts in mouse neonates would be of interest, as previous findings have suggested rejection under these circumstances (Mattis *et al.* 2014), which may have a detrimental effect on the potential for neonatal desensitisation.

Chapter 6

Improving graft survival in the mouse striatum

6.1 Summary

Although **Chapter 5** aimed to determine whether a method of neonatal desensitisation could be successfully applied in mouse hosts to achieve long term survival of primary human foetal tissue transplants, poor survival of human xenografts and mouse transplants prevented this from being investigated effectively. Consistent survival of neural transplants in the mouse host has been found to be a problem by others using a range of cells, including mouse PF tissue and ESC derived neurons. The aim of the work described in this chapter was to address these problems since the parameters for mouse donor cell survival need to be resolved before xenografts can be investigated further. Thus two key variables were explored that have been shown to be important for rat to rat transplant survival; the gestational age of donor cells and the cell preparation method. Four groups of mice were transplanted with different types of mouse PF tissue. Two different donor ages were compared (E12 and E14), to determine whether younger donor tissue would improve survival as well as graft phenotype according to the relevant Carnegie staging of mouse foetuses compared to rat. In addition two cell preparation methods were tested to identify whether the reduced trituration of tissue following digestion could improve graft survival and morphology as compared to heavily triturated quasi single cell suspensions[‡]. Good graft survival was found in all groups, although still not 100% as may be expected in rat tissue transplants in rat hosts. This implies either a difference between the rat and the mouse as transplant hosts, or an issue with survival of mouse donor tissue following transplantation.

[‡] Declaration
The experiment discussed in this chapter was carried out in collaboration with another PhD student; DJH. Data discussed here relates to CD-1 mice. An additional cohort of BL/6 mice underwent the same procedures and these data will be discussed as part of DJH's PhD thesis. Dissection of mouse tissue and the majority of dissociation and preparation of cells for transplantation were carried out by Ngoc-Nga Vinh (NNV).

6.2 Introduction

As discussed in **Chapter 5**, mice are desirable transplant hosts for a number of reasons, including the availability of genetic models of disease. However, as can be seen from the results of the experiments described in **Chapter 5**, survival of transplants of mouse tissue in mice is more variable than may have been expected. These findings are not exclusive to the experiments presented here; other data from our lab group shows difficulty in achieving success using mouse hosts, including with grafts of mouse tissue (unpublished data, Evans AE; Kelly CM) and examples of mouse grafts in the literature also tend to be small in comparison to those seen in the rat (El-Akabawy *et al.* 2012; Johann *et al.* 2007; Kelly *et al.* 2007). Without being able to achieve successful transplant survival it is not possible to test whether desensitisation can be induced in the mouse. Therefore this chapter aims to investigate whether modifications to the transplantation protocol will improve survival of mouse tissue transplanted into the mouse brain, before re-examining xenograft survival.

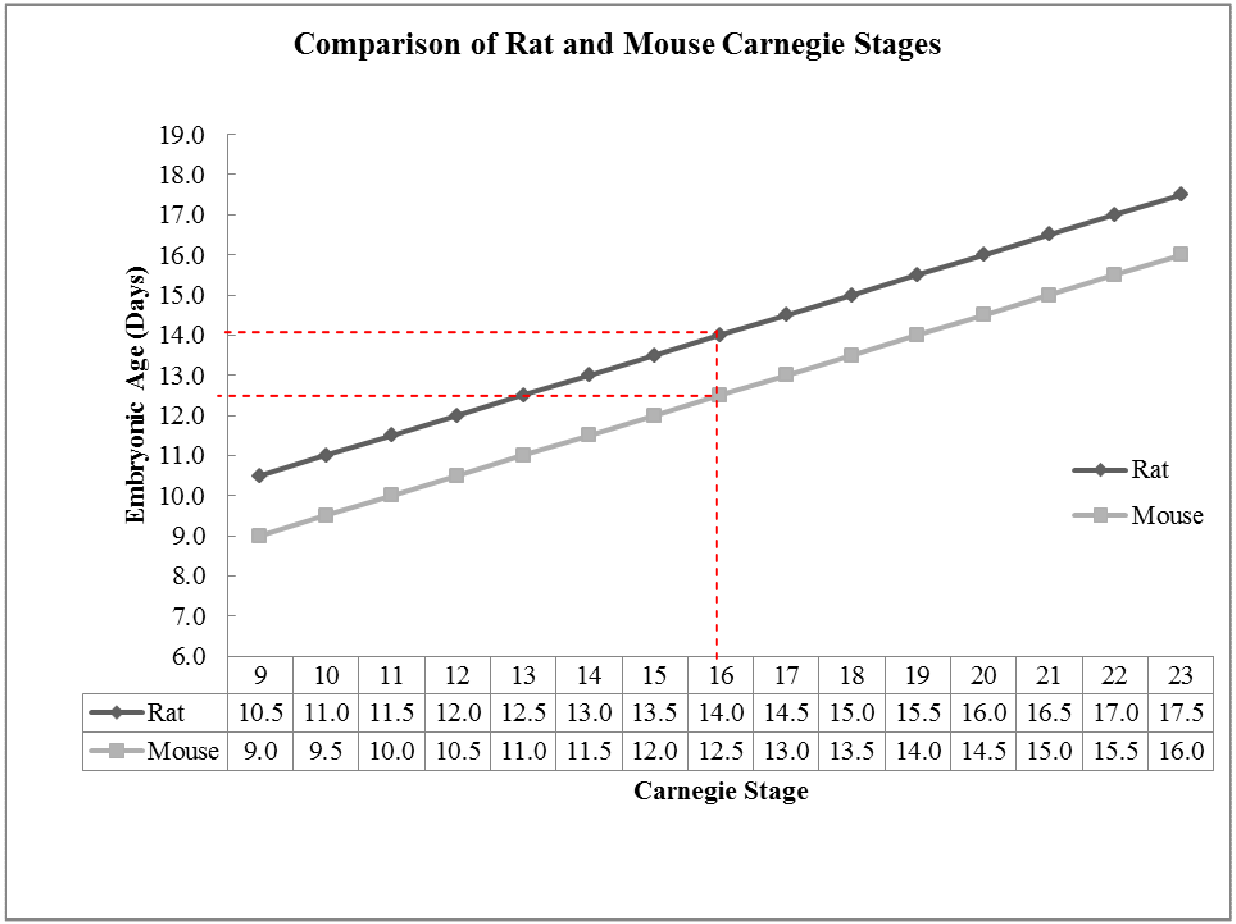
Many of the current conditions for preparation of donor cells for transplantation, including donor age, dissection parameters and cell preparation have been developed and optimised mainly for transplantation in the rat (Barker *et al.* 1995; Schmidt *et al.* 1981). This includes the foetal donor age used for transplantation of mouse tissue and the preparation of cell suspensions. For rat WGE transplanted into the rat striatum, donor tissue is dissected from the foetus at E14-15 in order to collect cells at the right point in striatal development so they will differentiate after transplantation into MSNs expressing DARPP-32 (Watts *et al.* 1997). Numerous experiments have shown this to be successful, demonstrating integration and functional improvement of E14-E15 tissue transplanted to the QA lesioned rat striatum (Brasted *et al.* 1999a; Brasted *et al.* 1999b; Döbrössy and Dunnett 1998; Nakao *et al.* 1998; Nakao *et al.* 1999). Additional studies have demonstrated similar recovery following lesion with alternative excitotoxins or metabolic toxins (see Dunnett *et al.* (2000) for a review). In the mouse, WGE from E14 tissue has also been used for striatal transplants, however due to differences in development between these species this may not be the optimum age of donor tissue to transplant. Comparing the Carnegie stages of development between mouse and rat, mouse

E12.5 corresponds to rat E14 (**Figure 6.1**). Although striatal transplants of donor tissue of varying donor ages have been compared in the rat (Fricker *et al.* 1997; Schackel *et al.* 2013), this has not yet been systematically explored in the mouse. It has been shown that even in rat grafts the use of younger donor tissue (E13) produces better behavioural improvements and more phenotypically accurate grafts containing a greater area of DARPP-32 positive 'P zones' (Schackel *et al.* 2013).

In addition to the effect of donor age, modifications to the preparation of cell suspensions have been considered with regard to rat transplants (Watts *et al.* 2000a), and in clinical trials, but not in the context of mouse tissue transplants. It has been concluded that transplanting tissue as a single cell suspension, rather than tissue pieces reduces the likelihood of rejection since tissue pieces contain intact donor vasculature which may contain donor APCs which activate host lymphocytes. This is thought to provoke a stronger host immune response than transplanting single cells (Chen *et al.* 2011). However; transplants of tissue pieces have been utilised in the clinic for both PD (Freed *et al.* 2001; Olanow *et al.* 2003) and HD (Bachoud-Lévi *et al.* 2000; Bachoud-Levi *et al.* 2000; Hauser *et al.* 2002; Kopyov *et al.* 1998). Post-mortem data from a number of clinical transplants in HD has shown survival of grafts, with some inflammatory infiltration though not sufficient to cause rejection of all graft deposits under variable immunosuppression regimes (Capetian *et al.* 2009; Cicchetti *et al.* 2009; Freeman *et al.* 2000; Keene *et al.* 2007).

This experiment therefore aimed to compare an earlier gestation than normally used, E12, to E14 mouse striatal grafts to determine whether transplants from E12 foetal tissue may promote better survival and yield an internal organisation more similar to that of the normal striatum. Additionally, we examined the effect of modifying the cell suspension in mouse grafts by comparing a standard single cell suspension (CS) as used in previous chapters to a "partial tissue pieces" style suspension where striatal tissue was digested but not triturated into a single cell suspension (referred to as "TP"). Although this did not involve transplantation of traditional "chopped" pieces of tissue, the reduction in trituration was intended to reduce the severity of treatment of tissue, and therefore improve donor cell viability and survival. This method has previously been demonstrated to yield greater functional improvements with grafts of rat GE to the QA lesioned rat striatum (Watts

et al. 2000a) and improved survival of human VM transplants (Rath *et al.* 2013). The resulting preparation consisted of small pieces of tissue which were only triturated sufficiently to allow the pieces to be drawn up into a syringe for transplantation. These conditions were compared at both foetal ages with the aim of determining the optimum transplantation paradigm to promote the best survival of mouse grafts. Modifications described in **Chapter 5** from findings from xenograft experiments were also included, with transplants delivered 10 days after QA lesions, and an increased number of cells used for transplantation. The findings from this experiment also aim to address whether poor graft survival in **Chapter 5** may be in part related to the treatment of the donor tissue, as opposed to being primarily due to the mouse host environment. The improvement of mouse tissue survival is required to demonstrate sufficient transplant survival in mouse hosts before re-addressing the issue of xenografting and the desensitisation of mouse hosts.



	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Rat	10.5	11.0	11.5	12.0	12.5	13.0	13.5	14.0	14.5	15.0	15.5	16.0	16.5	17.0	17.5
Mouse	9.0	9.5	10.0	10.5	11.0	11.5	12.0	12.5	13.0	13.5	14.0	14.5	15.0	15.5	16.0

Figure 6.1 Differences in Carnegie stages between mouse and rat during foetal development. The red dashed line indicates the foetal age used for striatal transplants in the rat (E14) and how this compares to the mouse. Data from Hill (2013)

6.3 Experimental Design

A total of 30 adult male CD-1 mice (>20g) were used in this study. All received QA lesions to the right striatum and 10 days later were split into one of 5 groups as shown in **Table 6.1** below. E12 partial tissue pieces preparations (E12TP) were transplanted as a pair of striatae, treated with trypsin for just 10 minutes and resuspended in DMEM/F12 to a total volume of ~4µl per transplant with minimal trituration. E14TP were prepared in the same way, this time transplanting one striatum per host. This was based on an aim to keep cell number consistent between transplant groups, since E12 WGE is significantly smaller than E14, and cell suspension groups were all transplanted with $5x10^5$ cells we reasoned that transplanting a smaller quantity of tissue at E14 would be more comparable. Both E12 and E14 cell suspensions were made up as in previous chapters and as described in **Chapter 2**. Mice in these groups received transplants of $5x10^5$ cells in 2µl DMEM/F12.

Condition	Number of Mice
E12 partial tissue pieces	7
E12 cell suspension	6
E14 partial tissue pieces	7
E14 cell suspension	7
Lesion only control	3

Table 6.1 Numbers of mice in each condition

6.3.1 Histology and Immunohistochemistry

12 weeks after transplantation, mice were transcardially perfused with fresh ice cold 4% PFA and the brains collected for histological analysis. Brains were sectioned as previously at 40µm and stored in anti-freeze at -20^{o}C. 1:12 series were mounted and Nissl bodies stained with CV, and further 1:12 or 1:6 series were stained for immunohistochemistry using NeuN (1:2000), DARPP-32 (1:4000), Iba1 (1:8000) and CD3 (1:500) as described in **Chapter 2**.

6.3.2 Quantification and statistical analyses

Graft volume was calculated by measuring graft areas on each section stained with NeuN on Image J and calculated with formulae described in **Chapter 2**. Total cell number was counted manually on Image J, and differences between graft volume and cell number were investigated by ANOVA. Immune marker staining was graded (0-4) and grades were compared between transplant groups with non-parametric Kruskall-Wallis tests.

6.4 Results

6.4.1 Graft survival at 12 weeks post-transplantation

Surviving grafts were identified in the first instance using CV staining to detect denser areas of staining thought to be typical of the presence of a graft. Numerous previous studies have shown grafted tissue to have a different organisation of CV compared to the surrounding host tissue. The presence of $NeuN^+$ cells was used to confirm neuronal differentiation. In the majority of cases the graft was placed entirely within an area denuded of neurons by the QA lesion, allowing NeuN staining to provide a good level of evidence that this corresponds to surviving transplanted cells. Transplants which were identified as $NeuN^+$ within the lesioned striatum were all counted with no minimum cut off for graft size. Although some grafts were smaller than others, there was little variability found within groups. The number of surviving grafts is outlined in **Figure 6.2** (A, B). In contrast to findings in previous experiments, relatively good survival of mouse transplants was found in all conditions, therefore the number and phenotype of surviving transplanted cells was quantified. Graft volume was estimated and total numbers of surviving neurons ($NeuN^+$) were counted and corrected with the Abercrombie correction. Comparisons of graft volume and the number of surviving cells between transplant groups can be found in **Figure 6.3**. Measurement of graft volume and DARPP-32+ patch volume is also displayed here. Photomicrographs of representative surviving transplants are shown in **Figure 6.4** and **Figure 6.5**.

A univariate ANOVA was run comparing the number of NeuN+ cells in the graft between the two donor age groups (E12 and E14) and two tissue preparation groups (TP and CS). This showed a significant effect of age; $F(1,16) = 5.516$, $p<0.05$, but not the method of cell preparation; $F(1,16) = 0.220$, $p = 0.645$. The same pattern was reflected in graft volume, with a significant difference found between E12 and E14 transplants; $F(1,16) = 6.025$, $p < 0.05$ but not between TP and CS; $F(1,16) = 0.061$, $p = 0.809$. These significant differences in $NeuN^+$ cells and graft volume are due to the presence of larger surviving grafts in those hosts receiving transplants from E12 donors (**Figure 6.3** A, C). Although transplants appeared largest in hosts transplanted with

E12 TP, there was no significant interaction between the effect of donor tissue age and cell preparation (Max F (1,16) = 2.974, p = 0.104).

6.4.2 Phenotype of surviving transplanted cells

Staining for DARPP-32 revealed the presence of mature MSN-like neurons in the surviving transplanted cells (**Error! Reference source not found.** and **Error! Reference source not found.**; I-L). The areas of DARPP-32+ patches (p-zones) within the graft area were measured to calculate their total volume in each transplanted animal (**Error! Reference source not found.**; D, F). The percentage of total graft volume occupied by these p-zones was then calculated for comparison between groups **Error! Reference source not found.** (E, F).

Univariate ANOVAs were again carried out to compare the number of DARPP-32^{+} cells in the transplant, and the volume of p-zones within grafts between the two donor ages and cell preparations. No significant effects of donor age ($F(1,16) = 0.546$, $p = 0.471$) or cell preparation ($F(1,16) = 1.742$, $p = 0.205$) were found in the number of D32+ cells. There were also no significant differences in patch volume between the two donor ages, however significantly larger areas of p-zones were found in transplants of TP than CS; $F(1,16) = 5.884$, $p < 0.05$ (**Error! Reference source not found.** B, D). Again no significant interaction was found between the effect of donor age and cell preparation (Max $F(1,16) = 1.548$, $p = 0.231$). Interestingly, the percentage of the grafts occupied by DARPP-32+ patches appears to be greatest in E14 TP transplants, although as this only consisted of 3 surviving grafts this cannot be confirmed.

A

Transplant group	Number of surviving grafts (%)	Mean graft volume (µm^3)	Mean number of NeuN$^+$ cells (x10^3)	Mean number of D32$^+$ cells (x10^3)	Average volume of p-zones within transplant (µm^3)	% of graft occupied by D32$^+$ patches
E12 TP	5 (85.7%)	2.9x10^8	18.5±7.5	4.6±3.8	1.4x10^8	49.0%
E14 TP	3 (42.8%)	1.5x10^8	7.6±3.2	4.1±0.5	1.2x10^8	79.3%
E12 CS	5 (83.3%)	2.3x10^8	12.6±6.1	1.7±1.0	0.4x10^8	17.4%
E14 CS	6 (85.7%)	1.9x10^8	11.0±4.0	4.0±1.8	0.8x10^8	39.7%

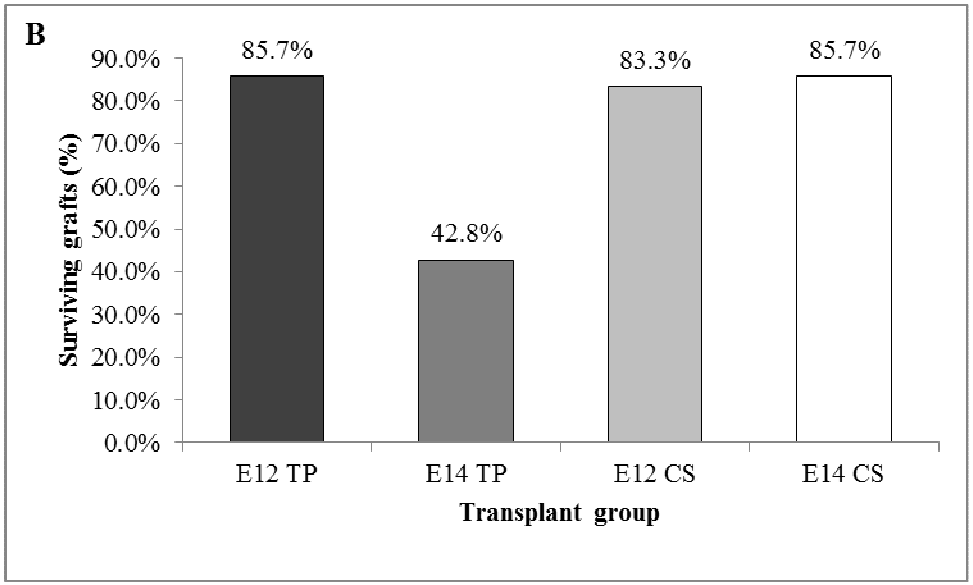


Figure 6.2 Surviving mouse transplants in CD1 mouse hosts

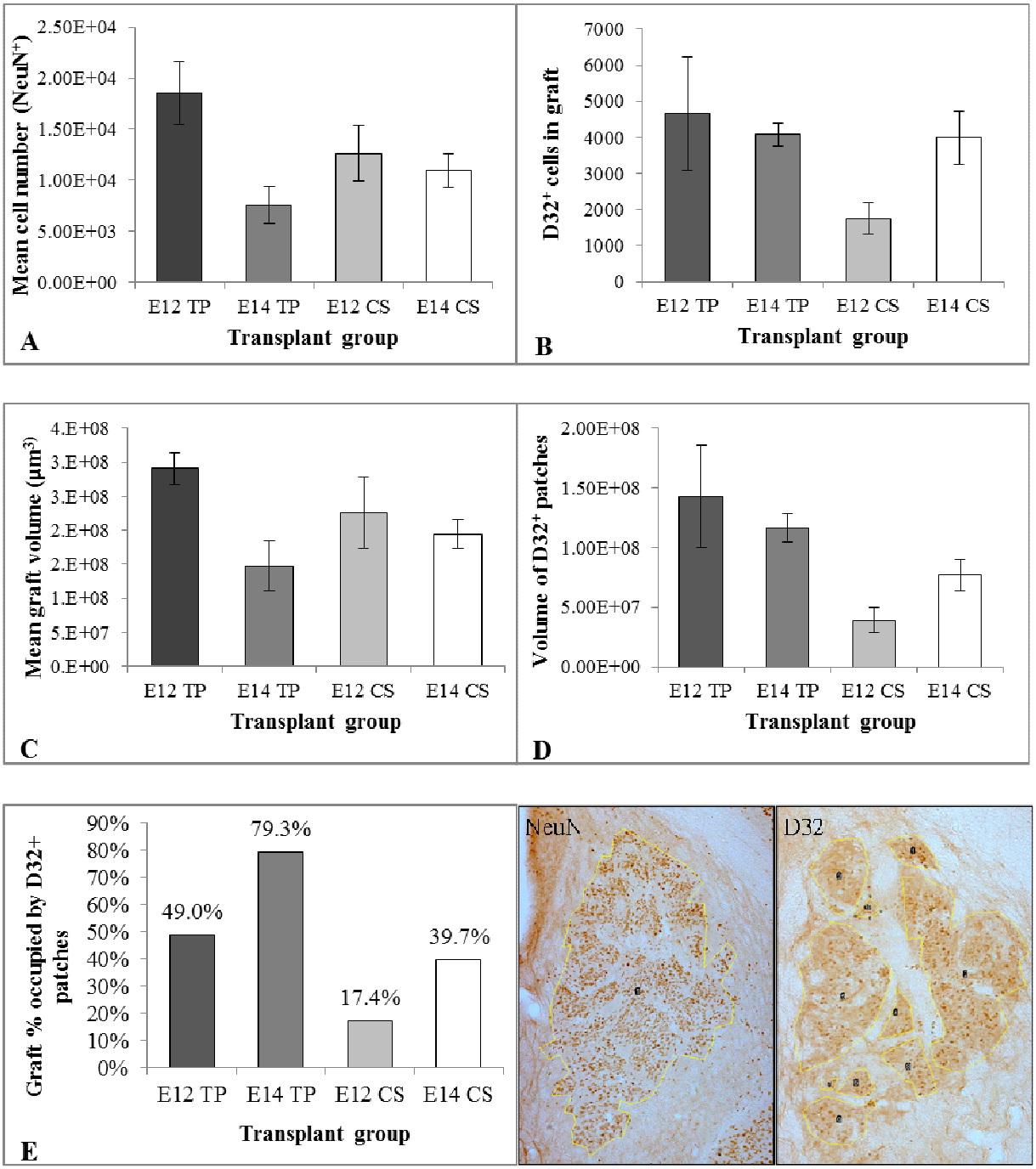


Figure 6.3 Mean total cell number of surviving cells in mouse grafts ($NeuN^+$, A), and number of $D32^+$ cells in the grafted area (B). Mean graft volume (C) and the total volume of D32 patches (p zones) in the graft (D). Percentage of total graft volume occupied by D32+ patches (E). Examples from Image J of measurements of total graft area and patch areas used to calculate total graft and patch volume (F). Marker indicates the centre of a p-zone and the yellow line is the boundary of the p-zone used in the volume calculation. TP = Tissue pieces transplants; CS = Cell suspension transplants

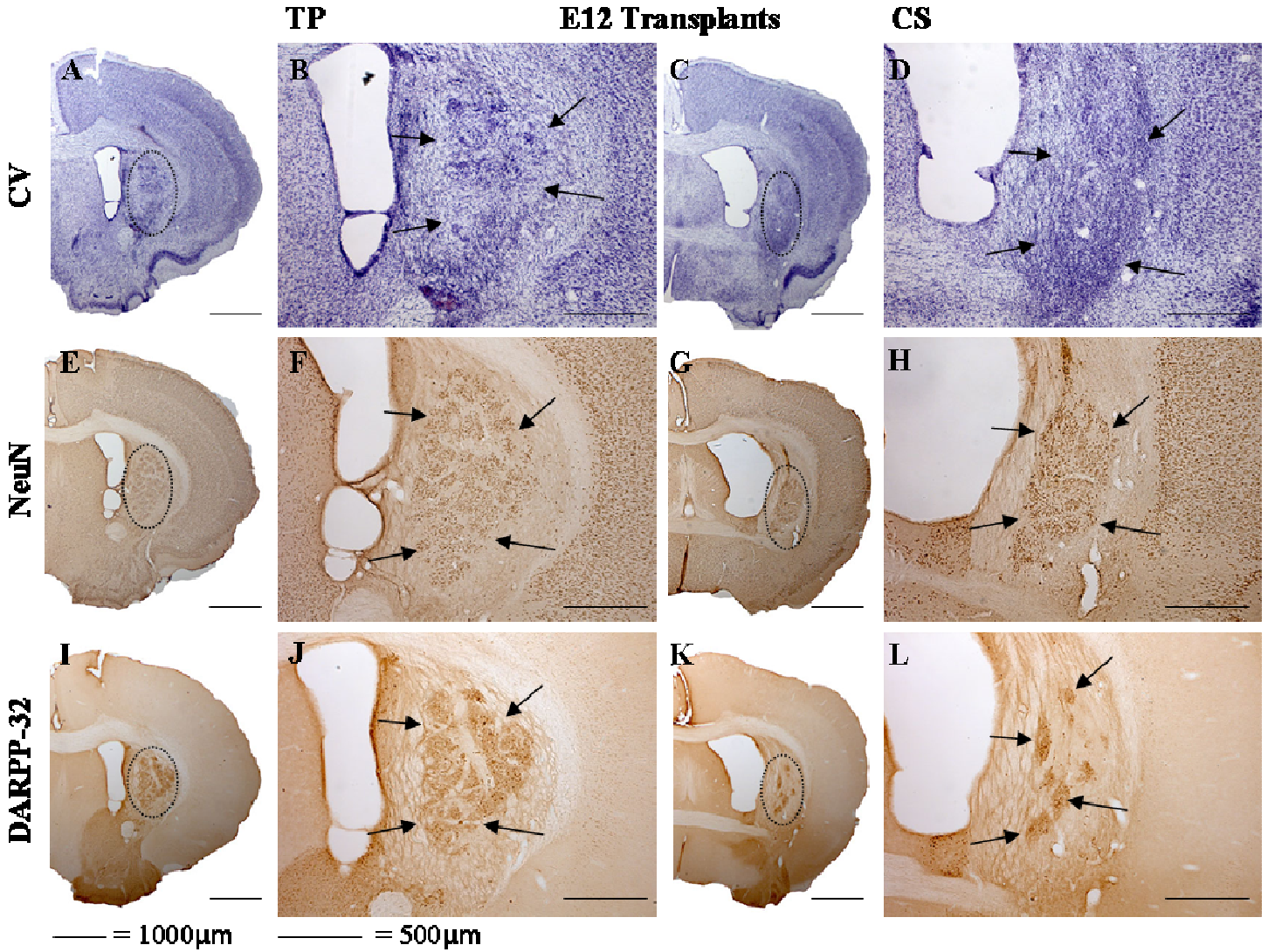


Figure 6.4 Photomicrographs of typical sections from mouse hosts transplanted with E12 mWGE and stained with CV (A-D), NeuN (E-H), and DARPP-32 (I-L). Donor tissue was prepared either as a tissue pieces style preparation (TP - left panels: A,B,E,F,I,J); or cell suspension (CS - right panels: C,D,G,H,K,L). Grafts are circled on low magnification images, and graft-host border is highlighted with arrows on high magnification images.

11

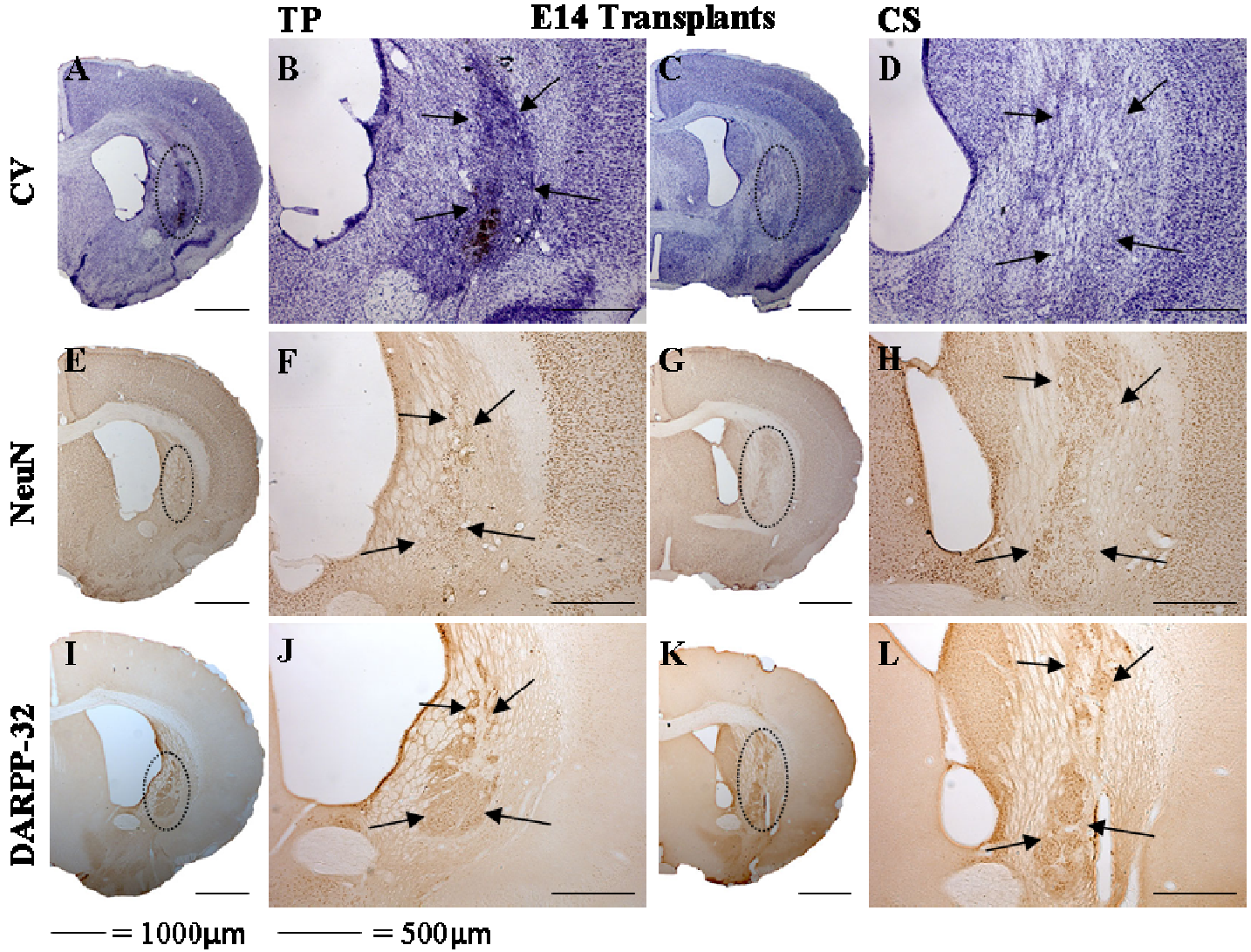


Figure 6.5 Photomicrographs of typical sections from mouse hosts transplanted with E14 mWGE and stained with CV (A-D), NeuN (E-H), and DARPP-32 (I-L). Donor tissue was prepared either as tissue pieces (TP - left panels: A,B,E,F,I,J); or cell suspension (CS - right panels: C,D,G,H,K,L). Grafts are circled on low magnification images, and graft-host border is highlighted with arrows on high magnification images.

6.4.3 Mouse host immune response to striatal mouse grafts

To assess the mouse host response to transplants of mWGE, sections were stained for the microglial marker Iba1. Sections were graded according to the rating scale described in Chapter 2. The highest grade assigned to a section for each animal was recorded, and these are presented by group in **Figure 6.6**. **Figure 6.7** shows representative Iba1 staining for each grade; grade 0 is not shown since no section received this grade. The microglial response in the majority of hosts was low, with staining in only a few animals showing greater immunoreactivity than that seen in lesion only controls. The data was analysed using a non-parametric Kruskall-Wallis test, showing no significant differences in grades of microglial staining between groups; $H(4) = 9.337$, $p = 0.053$.

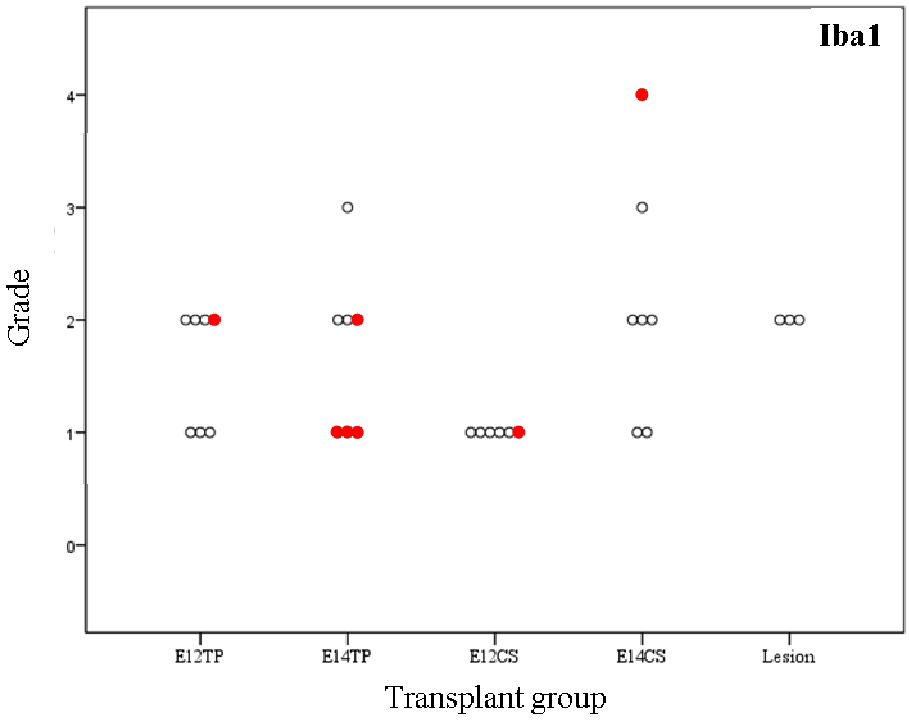


Figure 6.6 Grading of Iba1 immunoreactivity in each transplant host. Each circle corresponds to 1 mouse host; red circles denote rejected transplants. "E12TP" = E12 tissue pieces, "E14TP" = E14 tissue pieces, "E12CS" = E12 cell suspension, "E14CS" = E14 cell suspension, "Lesion" = Lesion only controls.

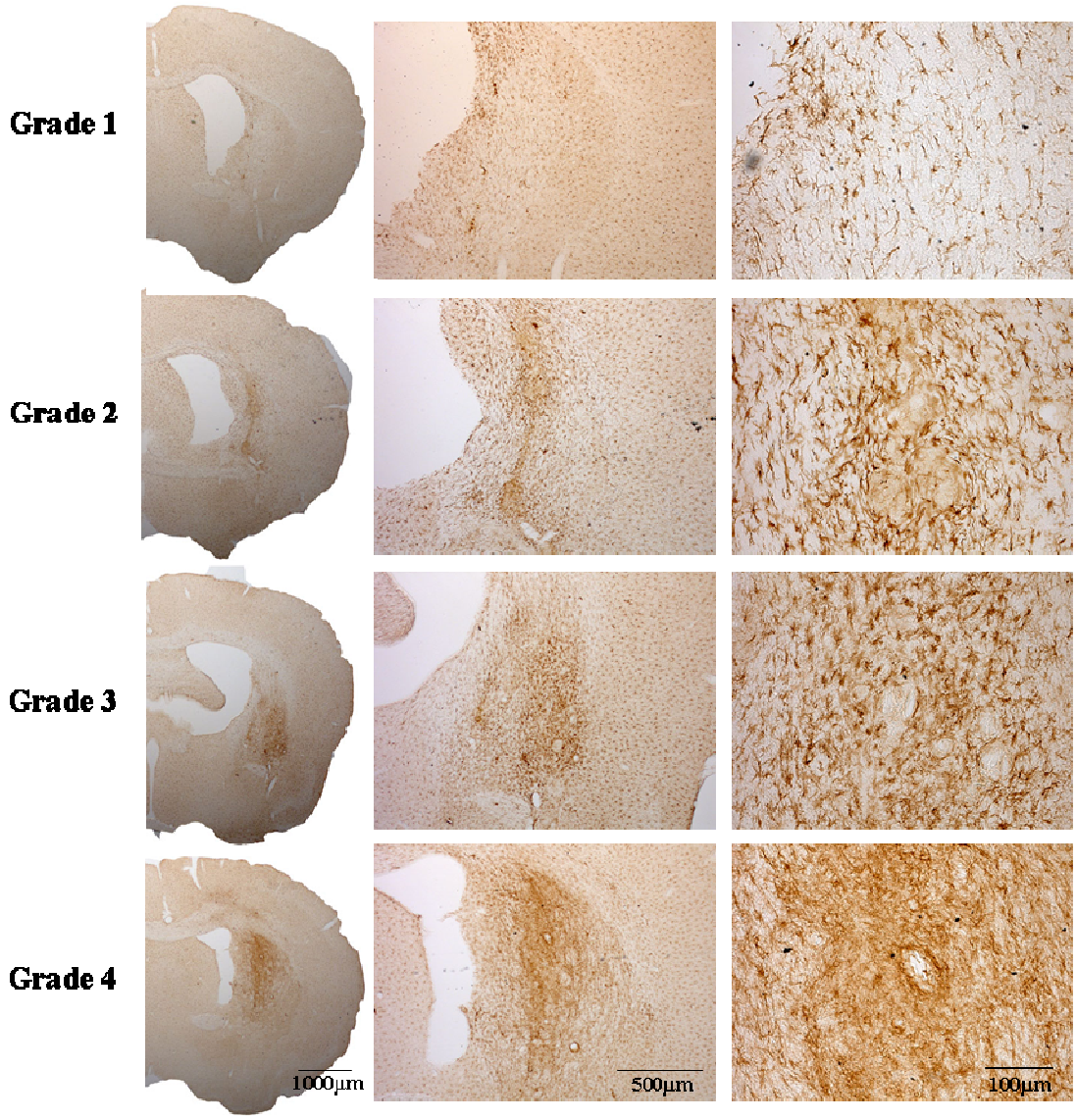


Figure 6.7 Examples of immune marker staining grades (1-4) for Iba1 in mice grafted with mWGE. Images for grades 1 and 2 were from hosts transplanted with E14TP and grades 3 and 4 from hosts transplanted with E14CS. Earlier grades mostly show the presence of resting, ramified microglia, with later grades showing increased density around the graft site with retraction of distal processes

6.5 Discussion

This chapter aimed to test the mouse striatal transplant protocol using mouse tissue and determine whether modifications to the cell preparation regime and variations in donor tissue age would improve the poor levels of survival observed in **Chapter 5**. CD-1 mice were transplanted with either a single cell suspension (CS), as used in previous mouse and rat transplant experiments or with a dissociated tissue pieces suspension (TP) where trituration of the cells was kept to a minimum. Additionally, both cell preparations were tested using two different foetal donor ages; E12 and E14 to determine whether survival may differ with the use of younger donor tissue. Due to the concerns with the reliability of CV staining for the detection of graft survival, discussed in **Chapter 5**, antibody staining with the mature neuronal marker NeuN was used to confirm the presence of surviving cells. With NeuN and DARPP-32 staining, the structure of surviving grafts could clearly be identified within the lesioned striatum.

In contrast to previous mouse to mouse transplants presented in this thesis (**Chapter 5**), good survival was found in all transplant groups in this chapter. It is not clear why survival should be improved in this experiment which used the same E14 cell suspension preparation as those in **Chapter 5**, where survival previously was so poor, although there are a number of potential reasons. It is possible that with experience, cell preparation and transplantation techniques were improved, increasing the likelihood of transplant survival. However this is unlikely since successful transplantation was readily achieved in rat hosts. Alternatively; the trypsin digest of tissue was modified slightly for this experiment, with incubation for 10 minutes rather than 20, as it was considered sufficient for dissociation of tissue. In transplants of VM tissue, in particular from mouse, it has been noted that the tissue is sensitive to prolonged enzymatic treatment (Breger *et al*, unpublished observations). Potentially this could have had a similar effect on the striatal tissue used here and explain the improved survival in this study as compared to those in **Chapter 5**. This would also agree with the poor survival of mouse to rat xenografts seen in immune compromised hosts in **Chapter 4.**

6.5.1 The effect of donor age

Donor tissue from two different foetal ages (E12 and E14) was transplanted into mouse hosts in this experiment. These ages were chosen based on previous protocols used for striatal transplantation in HD. Most transplant work has been carried out in the rat, with E14 identified as the optimum donor age based on the phenotype of surviving transplanted cells and functional recovery (Fricker *et al.* 1997). However using Carnegie stages, the comparable gestational age in the mouse would be E12.5, rather than E14, in order to procure tissue at the same developmental stage. This study therefore aimed to compare transplants from both donor tissue ages to determine whether the use of younger donor tissue would yield better graft survival, with larger grafts and more surviving cells of MSN-like phenotype. In addition to this study, another experiment has used E12 donor tissue prepared as a TP style preparation using the same method described here to transplant mWGE into a mouse QA lesion model, finding improved graft survival as compared to E14 CS (Unpublished data, Evans AE).

The results of the present study showed significantly larger graft volumes along with higher cell numbers in grafts of tissue from E12 WGE compared to those from E14 WGE as measured from NeuN staining of mature neurons. There was no interaction between donor age and cell preparation, although the largest surviving transplants were seen in hosts transplanted with E12 TP. These data are in line with the previous literature discussed, showing that E14 rat WGE grafts yield larger transplant volume than from donor tissue of a greater gestational age (Schackel *et al.* 2013), as well as agreeing with concurrent findings from experiments in this lab. Donor age has also been shown to have an effect on the phenotype of transplants, with reports of younger rat donor tissue yielding transplants with greater volumes of P-zones within the graft (Fricker *et al.* 1997). P-zones are representative of striatal-like tissue within grafts containing a heterogeneous population of cells (Campbell *et al.* 1995; Wictorin *et al.* 1989). The volume of P-zones within the graft has also been demonstrated to be strongly correlated with recovery on motor behaviour tasks (Fricker *et al.* 1997; Nakao *et al.* 1996). The improvement in graft survival found in this study is also logical given comparisons between gestational stages in rat and mouse foetuses. With regard to the phenotype of transplanted cells at 12 weeks;

mature MSN like cells were identified in all surviving transplants with DARPP-32 staining, however no significant differences were found when comparing the two donor ages.

Previous work has suggested an effect of donor tissue age on the survival of grafts, since younger tissue may contain less microglial precursor cells which can present donor antigen following transplantation (Brevig *et al.* 2008; Dalmau *et al.* 1997). This has been confirmed in the assessment of the rat host to transplants of mouse tissue, in which improved survival was found from younger donors (Zimmer *et al.* 1988). Additionally, WGE grafts from younger foetuses contain more proliferating cells would have normally gone on to populate the cortex (during normal development proliferating cortical precursors in the subventricular zone migrate through the WGE on their way to the cortical mantle, and are more numerous at E12 than E14). This could produce larger grafts but with an overall reduced proportion of striatal-like tissue, as may be the case here, with larger grafts seen from E12 donors, although potentially a higher percentage of $DARPP32^{+}$ patches from E14 transplants.

6.5.2 The effect of donor cell preparation

In addition to comparing the differences in transplant survival between E12 and E14 donor tissue, the cell preparation protocol was also investigated. Routinely, tissue for transplantation is dissociated by enzymatic digestion and trituration to produce a quasi-single cell suspension. The number of cells can then be calculated and standardised for each host. This method has been developed over a number of years and is successfully applied when transplanting into rat hosts. Although studies into the optimum cell preparation for rat transplant survival have been carried out (Watts *et al.* 2000a; Watts *et al.* 2000b), the same is not reflected in the literature for mouse transplants. Standard protocols have been developed directly from those used in the rat but without systematic validation in the mouse. One of the aims of this study was therefore to compare the survival of mWGE prepared as "tissue pieces" or cell suspension from both E12 and E14 donor tissue. For transplantation of E12 gently triturated TP preparations; a pair of striatae were transplanted, whereas in the E14 groups, just one was transplanted. The aim of doing this was to transplant approximately the same amount of tissue in each condition (E12 striatae are much smaller than at E14) and estimated cell counts suggest that 2:1 for E12 to E14 is reasonable. However, this is a difficult parameter to control, as E12 tissue is likely to have a greater proliferative potential, and currently there is no recognised way of adjusting for this or for potential differences in the vulnerability of the two cell populations at the two different gestational ages.

The two preparations used in this study both included trypsinisation of the mouse PF donor tissue, but were followed by different trituration conditions. CSs were prepared following the standard protocol, following the trypsin digestion with trituration into a quasi-single cell suspension. Trituration of TPs was kept to a minimum; sufficient to allow the pieces of tissue to be drawn up into the transplant needle. Thus the TP group described here is not identical to chopped tissue pieces preparations which have been used previously (Bachoud-Levi *et al.* 2000; Redmond *et al.* 2008). This condition does provide, however, a less harsh treatment of the mouse donor tissue than traditional CS preparation protocols (Rath *et al.* 2013; Watts *et al.* 2000a). In comparing surviving transplants between tissue preparation conditions in this study, no differences were found in graft volume or the number of

surviving cells. As mentioned previously, E12 TP produced the largest grafts, but since E14 TP yielded the smallest grafts there was no improvement reflected statistically with the use of tissue pieces over all. As fewer grafts survived in the E14 groups, firm conclusions cannot be made regarding these data, although a trend towards increased volume of DARPP32^{+} patches in TP preparations was seen. However when looking at the percentage of the total graft volume occupied by these patches, the E14 TP was the highest.

No significant differences were found between cell preparations in the number of DARPP-32+ cells in the transplants, however significantly larger areas of p-zones within the transplants were produced from transplanted TP. This may be due to the reduced trituration and disruption of striatal tissue, allowing better formation of striatal-like tissue within the grafts. The effect of cell preparation on survival, integration and behavioural recovery has been investigated recently in human to rat VM transplants (Rath *et al.* 2013). The authors found that VM transplants to the striatum of tissue pieces produced the optimum survival and reversal of rotational bias. In our experiment, the number of DARPP-32^{+} cells and P-zones developing within the grafts could be affected by minor differences in the dissection between donor tissues, since non-patch zones may also be dissected along with WGE. In general transplants from WGE alone yield 30-50% P-zones as a proportion of total graft volume, as compared to the lateral GE (LGE); producing 80-90% P-zones and the medial GE (MGE); 25% P-zones. Large variation between transplant groups is seen in this study in the percentage of total graft volume occupied by P-zones. This could suggest variations in dissection of tissues, although the extent of this is unclear, since animals receiving cell suspension grafts were transplanted with a cell suspension of tissue pooled from multiple donor foetuses, and in tissue pieces transplant groups; hosts were each transplanted with tissue from a different donor. However percentages could also be expected to vary dependent on donor age.

Although no behavioural testing was carried out on host animals in this study, previous data has shown a correlation between the level of behavioural improvement observed following transplantation and the volume of P-zones within the transplants (Fricker *et al.* 1997; Nakao *et al.* 1996; Schackel *et al.* 2013). This suggests that although some transplant protocols may yield larger surviving grafts, this may not be

the optimum protocol to offer functional improvement. Future work would be necessary to determine which transplant condition provides the best function. Data here are still suggestive of E12 TP as providing the optimum transplant condition regarding the size of graft and patch volume; however the percentage of the whole graft itself occupied by DARPP-32^{+} patches was found to be greater in the E14 TP group.

6.5.3 Mouse host immune response to striatal transplants of mWGE

Staining for the microglial marker Iba1 identified a consistent presence of microglia in and around the grafted area in sections from all groups. No differences were found in the extent of immunoreactivity, including in those animals which received lesions only, and no transplant. This shows that transplants of allogeneic tissue induce no more of a microglial response than a QA lesion and that no differences in the host immune response are found between different cell preparation groups. Previous studies have suggested that the transplantation of tissue pieces may provoke a stronger immune response due to the presence of intact donor vasculature and APCs (Chen *et al.* 2011). This has been reflected in reports of a stronger microglial response to VM transplants of tissue pieces from non-human primates into rat hosts, and a lack of optimal re-innervation of the host striatum (Redmond *et al.* 2008). However, although the TP group described in this study may not be comparable to this due to the differences in preparation, a number of clinical trials have used tissue pieces for transplantation with some reports of inflammation but without complete rejection of donor cells following variable immunosuppression (Capetian *et al.* 2009; Cicchetti *et al.* 2009; Freeman *et al.* 2000; Keene *et al.* 2007). Although an increased microglial presence may not necessarily be detrimental to the graft, with microglia also offering protective properties, quantification in these studies has used assessment of activated microglia which presents a different morphology to resting and is described in **Appendix 7.**

6.6 Conclusions

Data demonstrates that E12 tissue produces larger surviving grafts than E14 (as measured by NeuN counts and graft volumes), in line with previous data in the rat. This result could be anticipated given that this developmental stage of tissue would be expected to contain more proliferating striatal precursor cells than the later stage. Although no significant difference in NeuN counts was identified between the TP and CS preparations, TP preparations produced significantly larger P-zones, suggesting that they may produce more functional grafts. Further exploration of this is warranted to replicate these results and to empirically test the effect of age and cell preparation on behavioural function. Another important consideration that emerges from this chapter is whether the modifications to enzymatic digestion of tissue may be beneficial. The fact that graft survival improved across the board and yet the only modification that applied to all groups was the reduction in trypsin digestion time to 10, from 20 mins, suggests that this modification may be important and deserves further attention.

There was still a problem of graft variability within groups, which adds noise to the data. As TPs were prepared as individual striata (or pairs of), one aliquot of donor tissue was assigned to each host animal and all tissue pieces were drawn up into the syringe and transplanted. However following the regular protocol for cell suspension transplants, one “master” cell suspension was made up from which 2µl was taken to transplant each host. Although cell suspensions were gently agitated between transplants to prevent settling of cells it cannot be ruled out that hosts were transplanted with variable numbers of cells from this suspension and this could account for variability of graft size. For future transplant experiments it may be beneficial to prepare CSs as separate aliquots to ensure that the total number of cells is transplanted into each host. Further analysis would look at the profile of the immune response to different transplants in greater detail.

The findings in this chapter show that the mouse host can support good-sized transplants of mouse tissue if the conditions are right. Data from this experiment provide a solid basis for further work to improve the survival of xenografts in mouse hosts using a variety of immunosuppressive regimens, including testing the potential of the neonatal desensitisation method itself.

Chapter 7

General Discussion

Cell replacement therapy provides a novel treatment strategy for a range of diseases, including the neurodegenerative disorders PD and HD. To date, a large amount of pre-clinical data has provided evidence for the potential efficacy of this therapy with grafts of rodent tissue showing integration and function. The length of time required for differentiation of human donor cells and the need for adequate long term immunosuppression of rodent hosts has resulted in a limited amount of functional data for human primary tissue, and even less for alternative cell sources including human ESC and iPSC derived neuronal cells. The issues of immunosuppression complicate the long term assessment of these donor cells, in particular their functional potential. In order to test the safety and efficacy of human donor cells, we have proposed a novel method of promoting long term xenograft survival without conventional immunosuppression by use of neonatal desensitisation to xenogeneic cells. The experiments presented in this thesis aimed to further characterise and validate this method to allow its use in the prevention of rejection of novel human donor cell sources for CNS transplantation.

7.1 Is desensitisation specific to donor tissue type and species?

To date, neonatal desensitisation has been shown to be effective in the SD rat, with successful desensitisation to hPF cortical tissue, human foetal brain derived NPCs and hESC derived NPCs (Kelly *et al.* 2009b). Although this demonstrates the success of a novel method of promoting xenograft survival in the rat brain, further validation is required to allow routine use of the method in the testing of potential human donor cells for transplantation. The experiments discussed in **Chapter 3** of this thesis aimed to determine whether desensitisation with hPF donor tissue could be achieved with cells of a different source to those used for adult transplantation. On a practical level, the availability of human cells for injection of neonatal hosts is a constant limitation to the number of desensitisation experiments which can be conducted. Moreover, knowing whether cells must be from the same species as for the adult transplants is interesting in terms of beginning to understand the underlying mechanisms.

My initial findings suggested that transplants of hCTX could not survive following neonatal desensitisation of rat hosts with non-neural tissue, specifically with hSkin, with survival only reaching ~17% as compared to 66% in those desensitised with neural tissue. Further investigation aimed to determine whether this was due to the use of 'non-neural' tissue, or whether some feature of hSkin renders it unsuitable tissue for desensitisation. This was addressed by testing the potential for desensitisation in rat with a range of non-neural tissues. This experiment demonstrated successful desensitisation with both hLiver and hKidney, with 100% survival of hCTX grafts in these hosts, indicating that some tissue types may be more favourable for desensitisation than others. Reasons behind the unsuccessful desensitisation seen in hosts inoculated with hSkin as compared to those desensitised with hCTX, hLiver and hKidney are unclear since the mechanisms of the method have not yet been determined. Previous research into neonatal tolerance to allografts in mice has provided some potential suggestions as to why this may be the case (discussed below), however since neonatal desensitisation may not be assumed to represent tolerance the relevance of this is hard to determine. Studies have emphasised the relevance of the cell population injected neonatally, suggesting that the presence of mature APCs may result in an immunising effect rather than

tolerising (Matzinger 1994; Ridge *et al.* 1996). Thus potentially the large population of resident DCs in skin cells could have resulted in this type of immunising effect as opposed to successfully desensitising neonatal rat hosts. In contrast a neonatal injection of liver cells, known to contain populations of APCs which secrete immunosuppressive signals including IL-10, (Knoll *et al.* 1995; Sato *et al.* 1996) may be more likely to induce successful desensitisation.

A much lower than expected number of grafts was found to survive in hosts both desensitised and transplanted with hCTX (36%), compared to previous experiments to date (Roberton VH, unpublished observations, and Kelly et al 2009), although in the experiments described in **Chapter 4** 100% survival of hCTX transplants into desensitised rat hosts was seen. This highlights some variability in the successful desensitisation of hosts. As the mechanisms underlying the method are not known it cannot be determined which variables are important for consistent successful desensitisation of hosts. For example, if the number of cells injected is on the borderline of an effective dose, small changes could have a large effect. Additionally, potential differences in age of donor hCTX used for desensitisation between experiments may mean variability in the population of cells injected. As discussed in **Chapter 4;** younger donor tissue is thought to contain fewer microglial precursors which have the potential to act as APCs (Brevig *et al.* 2008; Dalmau *et al.* 1997). This may have a detrimental effect on the successful desensitisation of hosts as previously discussed.

Transplants of mWGE were not found to survive in this experiment, even in immunosuppressed controls, therefore success of desensitisation with mWGE could not be replicated as has previously been shown (Kelly *et al.* 2009b). However an interesting finding from this chapter was survival of at least 50% of hCTX transplants in hosts which were desensitised with mWGE. This finding was not anticipated and it is unclear why this would be the case, whether the neonatal injection has induced some kind of general reduction in the adult immune response to transplants, or whether desensitisation to some common epitope has been achieved. This experiment requires replication to confirm these findings and further studies to determine the underlying biology behind them.

In both **Chapter 3** and **Chapter 4** hCTX transplants were found to be very large at 12 weeks post-transplantation, in some cases resembling overgrowing transplants. Staining with Ki67 confirmed that some cells towards the core of the grafts were still proliferating at 12 weeks. Use of further staining methods are required to determine whether these are proliferating neuronal cells, as opposed to connective tissue or meninges which may have been dissected in error due to their close proximity to the cortical tissue being dissected. The experiments described here require replication with the relevant donor tissue type, in most cases WGE, to ensure that no differences in survival are due to the use of hCTX at a particular age. As found in **Chapter 4**, the donor age of the foetal tissue can affect the size and proliferation of surviving transplants, and the affect this has on survival has not been determined. It has been reported that the use of younger donor tissue may improve survival of transplants due to a lower number of microglial precursor cells which may act as donor APCs following transplantation (Brevig *et al.* 2008; Dalmau *et al.* 1997; Freeman *et al.* 1995; Zimmer *et al.* 1988).

7.2 Neonatal desensitisation to hCTX in mice

One of the main aims of the work presented in this thesis was to determine whether neonatal desensitisation to human neural xenografts could be successfully achieved in mouse hosts. This would be a valuable step; demonstrating the method to be successful across species, and allowing its use in transplant experiments in mouse models of a range of diseases unavailable in the rat. The ability to successfully desensitise mice would also allow the use of various Tg models with modifications to their immune systems in order to investigate the mechanisms of the method. Currently these models are not available to the same extent in rats, thus restricting the ability to investigate these mechanisms.

A number of experiments were carried out in **Chapter 5**, beginning with testing the original desensitisation protocol in mouse hosts (Kelly *et al.* 2009b). Poor graft survival in this experiment, including in mouse tissue transplant control groups, led to various modifications to the transplantation and desensitisation protocols in subsequent experiments to attempt to improve graft survival in control groups, and determine whether desensitisation could be achieved by modifying the original protocol optimised in rats (Kelly *et al.* 2009b). These included testing inoculation with varying numbers of hCTX cells in the neonatal period, and modifications to the transplant protocol itself; increasing the number of cells transplanted and reducing the time between QA lesion and transplant. Although it has been reported that delivering transplanted cells at a shorter time post-lesion may improve the survival due to the host environment at this stage (Döbrössy *et al.* 2011; Johann *et al.* 2007), no improvements in graft survival were found in transplants delivered 2 days, 7 days, or 10 days after QA lesions, or indeed into the unlesioned striatum. Graft survival was poor across all groups, including those treated daily with the conventional immunosuppressant CsA.

Although successful desensitisation was not demonstrated in these experiments, since grafts in control animals, transplanted with hCTX and immunosuppressed with daily CsA injections, did not survive to greater than ~50% it cannot be determined whether this was due to the poor graft survival observed overall. However when compiling the data from all experiments conducted, the findings suggest that desensitisation does not improve survival to any greater degree than that found in

untreated hosts (~15% in contrast to 50% in CsA treated hosts). However the fact that graft survival was poor even with blocking of the T cell response with CsA is suggestive of some difference in the mouse brain as a host environment to that of the rat. Although it cannot be demonstrated with certainty that the loss of transplants was due to rejection, it is most likely that this is the case since up to 50% of mouse grafts and human xenografts in CsA treated hosts were found to survive, demonstrating that donor tissue could survive under some circumstances, albeit to a lesser extent than expected.

Much work has been carried out to characterise the response to neural xenografts in rat hosts transplanted with mouse (Finsen *et al.* 1991), porcine (Armstrong *et al.* 2001; Larsson *et al.* 2000) and human tissue. Some work by Larsson *et al* has aimed to understand the rejection responses to porcine tissue transplants in mice (Larsson *et al.* 2001a; Larsson *et al.* 2002; Larsson *et al.* 1999; Larsson *et al.* 2001b), however less detail is available concerning human tissue transplants. These investigators have suggested a difference in the immune response to neural xenografts between mice and rats; with a faster, more severe response observed in mouse hosts (Larsson *et al.* 2000). Moreover it has been reported that the use of high doses of CsA is required to promote xenograft survival in mice, although due to side effects this cannot be tolerated by the animals for long, prompting the authors to seek alternative methods, such as co-stimulatory molecule blocking (Larsson *et al.* 2002; Larsson *et al.* 2000). The findings of the work presented in this thesis, using human rather than porcine PF tissue support the notion that there is a difference in the response to neural xenografts between rats and mice. There is an indication of a strong microglial response to human xenografts in the mouse brain, as found in this thesis and by other groups (Mattis *et al.* 2014), although the precise differences between the two species has not yet been fully characterised.

Other groups have attempted to desensitise mouse hosts to human tissue, with little success (Janowski *et al.* 2012). These authors attempted to desensitise rat and mouse hosts to two different human cell types; an immortalised luciferase expressing glial restricted precursor cell line (hGRP) and a neural stem cell line derived from human cord blood and found poor survival in all conditions. Desensitisation with hGRP cells allowed detection of the cells *in vivo* with bioluminescence imaging

(BLI), showing that grafts were lost after two weeks in both desensitised and untreated mouse hosts. This was shown to be due to rejection of the cells, via dense infiltration of $CD45^+$ immune cells. Transplants into *rag2*$^{-/-}$ mice were shown to survive up to 3 weeks post-transplantation, but no longer time-points were assessed, thus no confirmation was demonstrated that their donor cells were capable of surviving in the long term following transplantation into the rodent brain. Survival of donor cells in immunocompetent hosts with the use of a conventional immunosuppression regime was also not shown (Janowski *et al.* 2012). Interestingly, BLI showed rapid signal loss following neonatal i.p. injections (Janowski *et al.* 2012). It is not clear how long cells injected to induce desensitisation in rats are present in the neonatal host, although it has been suggested that for tolerance induction, as with the natural development of "self" tolerance, the persistent presence of donor or "self" cells are required during the development of the immature immune system.

Other studies have suggested that neural xenografts do not survive in mouse neonates (Mattis *et al.* 2014), in contrast to the known survival of these transplants in rat neonates (Englund *et al.* 2002). If this is the case, then it appears that xenogeneic donor cells are rapidly rejected in both the CNS and the periphery of neonatal mice. This presumably would prevent the persistence of donor antigen during immune system development in the mouse host, and if this were the mechanism through which desensitisation is achieved then this could explain the difficulty in demonstrating desensitisation in this species. Assuming this to be so, then it may indicate that the immune system develops quicker in the mouse host than the rat and is more developed by birth, or is more equipped to reject the presence of human donor cells. However, until the issues of reliable transplant survival in adult immunocompetent mouse hosts are resolved it cannot be determined whether the neonatal desensitisation method can be modified for successful application in mouse hosts.

7.3 Transplants of mouse tissue to the mouse striatum

Experiments using xenografts of human tissue in **Chapter 5** allowed the identification of a general problem of graft survival in mouse hosts. These experiments included some hosts receiving mouse tissue transplants in order to ensure graft survival could be achieved in these conditions. Although some survival was observed, this was far lower than would be expected from neural transplants of rat tissue in rat hosts, which generally survive reliably without immunosuppression (Marion *et al.* 1990). As graft survival proved to be a limiting factor in the examination of the desensitisation method, this required further investigation of transplant protocols in mice.

Chapter 6 describes a mouse experiment designed to investigate whether poor survival of mouse tissue transplants in **Chapter 5** could be improved with alternative preparation of donor tissue, or with the use of tissue of a different donor age. A systematic experiment was designed to compare modifications to these variables with the standard protocol of transplantation of E14 quasi single cell suspensions. The rationale for using a different donor age was that the optimal gestational age for WGE in a rat transplant is around E13-15, with E14 being the most commonly used age which corresponds more closely to E12.5 in the mouse. The rationale for testing a different cell preparation was that digested tissue pieces improved survival of human VM grafts transplanted into rats (Rath *et al.* 2013), and that improvements on behavioural tasks are greater in rat WGE grafts (Watts *et al.* 2000a), therefore supporting the rationale for investigation of this preparation in **Chapter 6**.

Two cell preparation methods were used, a trypsinised “tissue pieces” style suspension (referred to as TP) in which a 10 minute trypsin digest was followed by a minimal trituration to leave small pieces of tissue rather than single cells, as described previously for preparation of rat LGE (Watts *et al.* 2000a) and human VM (Rath *et al.* 2013) and a standard trypsinised quasi-single cell suspension (referred to as CS) prepared using our standard trituration procedure. In order to allow comparison between the trituration parameters, the CS preparation was also subject to a 10 minute trypsin digest, which is shorter than the usual digest of 20 minutes. It is important to emphasise that the preparation labelled as TP in this thesis is not the

same as "tissue pieces" preparations used in clinical studies in which tissue is not trypsinised but is manually chopped into small pieces (Bachoud-Levi *et al.* 2000).

CD-1 mouse hosts received transplants of mWGE derived from either E12 or E14 donor embryos prepared as CS or TP, as described above, to systematically determine whether improvements in graft survival could be achieved. Initial analysis revealed better survival universally than in previous experiments. Findings were suggestive of a higher percentage of graft survival and larger graft volumes in transplants derived from E12 donors, irrespective of cell preparation type. However the different cell preparation groups did not result in differences in graft volume or phenotype, with no significant difference in the number of DARPP-32^{+} cells or volume of patches. Although differences were not significant, a trend towards a higher volume of DARPP-32^{+} patches in transplants from TP preparations was observed. When examining the percentage of the graft as a whole that was occupied by these patches, E14 TP transplants appeared to have the greatest; 79% of the graft as compared to 49% in the next highest group (E12 TP). These data require replication since this group contained only 3 surviving grafts and investigation of function is necessary to determine what graft composition produces the greatest functional benefit.

Good graft survival was seen in all transplant groups, including those hosts receiving E14 cell suspension transplants; the standard cell preparation protocol used for all mouse tissue transplants in **Chapter 5.** Although this may seem to conflict with the data from **Chapter 5**, in addition to the group differences in donor age and cell preparation, all tissue dissociation carried out in **Chapter 6** was conducted with a 10 minute trypsin digestion, as opposed to the 20 minutes used previously. This was in order to allow sufficient digestion for dissociation of the cell suspension groups into single cell suspensions, without breaking up the "tissue pieces". There has also been a suggestion of improvement in rat VM transplants which also used reduced digestion times in trypsin (Breger, L. Unpublished data). This could explain why graft survival was greatly improved generally in comparison to **Chapter 5**. Indeed, this may explain findings from **Chapter 4,** in which no surviving mouse transplants were detected in rat hosts, even in hosts which had been neonatally desensitised to mouse tissue or treated daily with CsA.

These findings lead to a conclusion that the mouse as a transplant host may not have been the only difficulty faced in **Chapter 5**. Since mouse graft survival in **Chapter 6** was fairly consistent across groups, and surviving transplants were of a good size, it is clear that transplants can survive in the mouse striatum. These data are suggestive of issues with the mouse donor tissue itself, which may be more sensitive to harsh cell preparation protocols than rat tissue. Reducing the trypsin step in the dissociation protocol, and perhaps the harshness of trituration of the tissue may prevent poorer tissue integrity. No differences in the immune response to transplants of mouse tissue were found in **Chapter 6** across the different donor age or cell preparation methods, suggesting no one preparation induces a stronger response than any other; therefore it is possible that the integrity of the donor tissue itself also has a detrimental effect on graft survival.

7.4 Methodological issues

7.4.1 Identification of mouse donor cells

A problem faced in **Chapter 5** was in the identification of mouse transplants in the mouse host brain. Without prior labelling of the cells, there is no way to clearly identify tissue of graft origin other than basing measurements on the presence of a deposit of cells within the lesioned striatum. In the case of **Chapter 6** lesions were quite clear and grafts were large enough to clearly be identified. However in **Chapter 5** this was not the case, with surviving grafts either being much smaller in size, or lesions producing insufficient cell loss to distinguish between graft and host. Additionally, due to the loss of tissue sections in these studies only CV staining could be used to identify surviving grafts. Although valuable in transplants in rats for initial identification of graft survival, and other aspects of graft morphology, it is clear that a large amount of staining may also be attributed to immune cells surrounding or infiltrating the graft. Further staining with neuronal and striatal specific markers is required to confirm the presence of surviving grafted cells within the lesioned striatum in the absence of a donor specific label. The lack of label for mouse cells also caused issues in the analysis of **Chapter 4**, as there was no clear way to confirm the absence of mouse donor cells in the rat brain. Although the only staining seen on CV stained sections from this chapter resembled a needle tract, the possibility of a few remaining mouse cells could not be ruled out as no confirmatory staining could be carried out. Since the hosts were not lesioned, no staining could be performed as in **Chapter 6** to distinguish between graft and host. Previous studies have used M2 and M6 antibodies, reported to be specific to mouse neurons and glia respectively (Olsson *et al.* 1997), however although these antibodies were not used in this study they have been reported in the literature (and confirmed in our laboratory) not to be specific to mouse. In addition the specificity of these two antibodies has been questioned with overlap being reported in detection of neurons and glia.

7.4.2 Subjective quantification of immune marker staining

To assess the immune response to striatal transplants in this thesis, immuno-staining was conducted for various immune markers. The variability in this staining,

dependent on the immune response between sections within one experiment makes manual quantification a challenge. The staining in one host may require counting only a few cells, whereas another might have a dense infiltration of cells which would require stereological analysis for adequate quantification. In order to avoid the problem, an established subjective rating scale was used, which has been extensively employed in the analysis of the immune response to neural transplants (Armstrong *et al.* 2001; Duan *et al.* 1995; Larsson *et al.* 2001b). In this analysis all sections from a series (e.g. 1:6/1:12) are graded for each host animal, the highest grade scored is then assigned to this animal for comparison with the rest. Although adopted as the best current standard, this potentially is not the most fair representation of the host response, since one section may contain a high grade of scoring resulting in that host being assigned a high grade, whereas another may have a lower grade, spanning a much larger amount of sections and therefore area of the brain, but resulting ultimately in a lower score. Alternative analysis such as optical density or automated counting would provide a more objective method of quantification. These methods are currently being optimised for future analyses.

7.4.3 Transplant host strains

With the exception of the C57BL/6J mice used in **Appendix 7** all the transplant hosts used for the experiments presented in this thesis were from outbred stock (CD-1 mice and LH/SD rats). SD rats have been used historically for successful neural transplantation experiments (Björklund and Stenevi 1979) and are still commonly used as transplant hosts today. LH rats have been subsequently selected for transplantation experiments which demand more thorough behavioural assessment of graft integration and function due to their inquisitive nature, and because the albino SD rats are known to possess lower visual acuity (Prusky *et al.* 2002). However unlike inbred rats which are genetically identical within colonies, removing variation at the MHC locus, outbred stocks are highly variable. Inbred strains allow for greater phenotypic uniformity and increase the potential to detect biological effects within a cohort of animals (Kacew and Festing 1996). Using outbred stocks for transplantation experiments increases the variability within groups, and may reduce survival of rat transplants as well as having an effect on survival of human transplants.

In some of the experiments described in this thesis, variability has been observed in survival rates in hosts desensitised with the same tissue types between experiments. In **Chapter 3,** for example, only 36% of hCTX transplants were found to survive in hosts desensitised with hCTX as compared to 100% in **Chapter 4**. Since hosts are desensitised to specific tissue types within litters to remove the need for identification of individual rat pups, there is no way to control for variability between these litters in outbred stocks. Although there are likely to be broad genetic differences between host animals, more similarities are likely to exist within litters, therefore one litter may be more amenable to graft survival than another. For the investigation of neonatal desensitisation in rodent hosts it would be valuable to use an inbred strain, where one source of variability in the data could be reduced, as control of human donor tissue haplotype is not possible. Unfortunately there is no obvious candidate for an inbred rat strain which would be ideal for transplantation and behavioural testing. If the method were to be optimised in an inbred strain, one which was suitable for behavioural assessment of graft function would be ultimately required.

7.5 Conclusion and future work

Although there is a need for replication of the findings discussed in this thesis, the data presented here has provided valuable validation of the neonatal desensitisation method in rat for its use in preclinical models for transplantation in neurodegenerative disease. The novel finding that hosts can be successfully desensitised using non-neural tissue types will allow desensitisation to be carried out with peripheral tissues, reserving neural tissue for transplantation. Further analysis will be needed to elucidate the reasons for this finding. The survival of human transplants in hosts desensitised to mouse tissue raises interesting questions about the specific mechanisms involved in neonatal desensitisation.

With regards to desensitisation in mouse hosts, although this has not been proven one way or another, the experiments presented here have revealed issues with both the mouse host and mouse donor tissue in transplantation experiments and the resolution of these issues will now allow directed modifications to the desensitisation protocol to determine why it has been unsuccessful so far and whether it can realistically be achieved. Addressing these issues with mouse tissue transplants has primarily provided potential improvements to transplantation protocols into mouse hosts in terms of tissue preparation and the improvement of the resulting phenotype of transplants, which can now be applied to improve survival of human xenografts in mouse hosts. The logical progression for further investigation in these areas is discussed here.

7.5.1 Rat

The experiments from both **Chapter 3** and **Chapter 4** require replication to confirm findings. Due to the long duration of most of these experiments (commonly 9-12 months from the time of neonatal desensitisation to the analysis of grafts) it was important to carry out experiments in parallel and thus it was not always possible to fully assess one experiment and replicate finding before addressing another question. Furthermore, whilst every effort was made to ensure group sizes would be sufficient for meaningful analysis to be carried out, I was limited by; litter sizes (which varied from 10-15 pups in the rat and 8-12 in the mouse), the availability of human tissue, and the less than 100% accuracy of the method. Hence some experiments fall short

in completeness due to resulting small group sizes. For **Chapter 3** it is necessary to increase numbers of hosts desensitised with non-neural tissue types to confirm that this successfully promotes survival of human neural xenografts, specifically to hPF WGE transplants, as opposed to cortex. Furthermore, to understand this finding further, it would be beneficial to further immunologically characterise the population of cells injected neonatally, and what disparity there may be between those which induce successful desensitisation as opposed to those which do not. This could be carried out by fluorescence activated cell sorting (FACS) analysis of donor cell suspensions to identify populations of, for example, antigen presenting cells, proliferating cells, and cells of a hematopoietic lineage. Populations of cells could be confirmed with immunocytochemistry for these markers on fixed primary donor cells. The experiment described in **Chapter 4,** to determine whether desensitisation is specific to the species of donor cells injected, requires replication to determine whether survival of human transplants in hosts desensitised with mWGE persists in comparison to untreated controls, and with transplants of hPF WGE instead of hCTX. These experiments would be conducted following optimisation of the preparation of mouse donor tissue discussed in section 7.5.2.

As well as defining the parameters for the desensitisation method, it is important to understand the mechanisms that underlie it. An experiment will attempt to investigate these mechanisms by testing organ samples from desensitised hosts using PCR and Western blotting, at various time points following neonatal injection for the presence of human genes/protein. Furthermore, it would be valuable to study additional characteristics of desensitised hosts such as the induction of peripheral chimerism, since this has been shown previously to be correlated with the successful induction of neonatal tolerance to allografts in mice (Borenstein *et al.* 2004; Chan *et al.* 2007). Optimisation of techniques has been achieved alongside the transplant experiments presented here. This work can now be carried out, and will provide data regarding the mechanisms involved in neonatal desensitisation to add to the characterisation reported in this thesis.

Following the finding that desensitisation can be achieved with tissue of a different type to that used to transplant the adult host; it is necessary to confirm that desensitisation can also be sufficient to promote long term survival of hESC and

hiPSC derived neuronal cells. Long term assessment of the success of the method is also required to demonstrate functional benefit in desensitised hosts with hPF tissue, and novel cell sources if possible. To this end experiments will aim to demonstrate this; with desensitisation of neonates which will host transplants of hESC derived MSNs or hPF WGE and undergo a battery of motor behavioural tasks to identify any functional improvements after transplantation. This has not yet been demonstrated since conventional immunosuppression does not allow such long term assessment of human graft potential.

7.5.2 Mouse

Findings from this thesis identified problems with rejection of human xenografts in the mouse brain, even under conventional immunosuppression. The recent finding that human xenografts are also rejected in neonatal mice implies there are differences between rat and mouse immune responses to neural transplants (Mattis *et al.* 2014). Therefore, future work aims to address this by comparing the rat and mouse host immune response to human transplants at various time-points post-transplantation. At present, a pilot experiment is being analysed, in which untreated or CsA immunosuppressed mouse and rat hosts received transplants of hCTX and were sacrificed 10 days post-transplantation in order to characterise the immune response to xenografts. Additionally, I aim to replicate the findings reported by Mattis *et al*, by transplanting human cells into neonatal mice of various post-natal ages comparing the response with that of the rat, or immune compromised mouse neonates (Mattis *et al.* 2014). The findings from this study will give insight into the problems faced with attempts to desensitise mouse hosts neonatally to human tissue, since a more severe host immune response in the neonatal period may prevent successful desensitisation.

As well as addressing problems identified with the mouse host, ongoing work also aims to resolve potential issues with the preparation of mouse donor tissue. Since findings from **Chapter 4** and **Chapter 6** are suggestive of sensitivity of mouse tissue to prolonged enzymatic treatment or trituration of donor tissue, an ongoing experiment aims to compare the effect of various treatments on the viability and development of cells *in vitro*. Subsequently, this will then be characterised further *in vivo*, with both human and mouse tissue to determine the optimum cell

preparation protocol for delivery of neural transplants. Although this has been addressed previously and optimised for rat transplants (Schmidt *et al.* 1981), the findings in this thesis suggest that further optimisation may be beneficial to promote survival of mouse and human donor tissue, in particular in the mouse host. Finally, the dissociation of tissues and cells for potential application in the clinic requires the use of animal-free reagents, therefore optimisation of the use of products such as TrypLE (Invitrogen) rather than bovine trypsin is required.

7.6 Final conclusions

The work presented in this thesis provides further validation of a novel method of promoting xenograft survival in the adult rat brain without the use of conventional immunosuppression. A method to achieve long term xenograft survival in the healthy rodent brain is crucial and is achieved by this method. It has been demonstrated that the rat can be desensitised to human donor tissue using various tissue types, sufficient to promote survival of hPF CTX up to 12 weeks after striatal transplantation. These experiments provide further validation for the method and for its use in the long term assessment of donor cells.

These experiments also highlight an issue with striatal transplantation into mouse hosts, in particular with xenografts. Much work is conducted under the assumption that transplantation protocols can be directly translated to mouse hosts; however I have found this not to be the case. I propose that CNS transplantation in mice requires more detailed investigation, in particular to investigate the rejection response to neural xenografts, and additionally the treatment of mouse donor tissue for transplantation. These studies are required before further comment can be made as to whether neonatal desensitisation can be successfully achieved in mice.

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Appendix 1

Reagents and suppliers

Reagent	Supplier	Address
Cyclosporine A (Sandimmun)	Novartis	Hampshire, UK
DMEM-F/12	Gibco	Paisley, Scotland
Diazepam	CP Pharmaceuticals	Wrexham, UK
DNase	Sigma	Poole, Dorset, UK
Euthatal	Merial Animal Research	Harlow, Essex, UK
HBSS	Gibco	
Isoflurane	Primaral Healthcare	Northumberland, UK
Metacam	Boehringer Ingelheim	Ingelheim, Germany
Penicillin Streptomycin	Gibco	
Trypan blue	Sigma	
Trypsin	Worthington	Freehold, New Jersey, USA
Trypsin inhibitor	Sigma	

Company	Location
Abcam	Cambridge, UK
AbD Serotec	Oxford, UK
BD Pharmingen	Oxford, UK
Harlan	Bicester, UK
Invitrogen (Molecular Probes)	Paisley, UK
Millipore	Molsheim, France
Santa Cruz	USA
Vector	Peterborough, UK
Wako	Neuss, Germany

Appendix 2

Recipes

Tissue dissociation

Cell suspension Media

DMEM-F/12

1% Penicillin streptomycin

DNase solution

0.05% DNase

In HBSS

Trypsin solution

0.1% trypsin

0.05% DNase

In HBSS

Perfusion and tissue storage

Prewash Buffer (PBS-1L)

18g di-sodium hydrogen phosphate

9g sodium chloride

1L distilled water

pH 7.3 (orthophosphoric acid)

Paraformaldehyde (PFA) solution (1L)

15g PFA (1.5%)/40g PFA (4%)

1L Prewash buffer

Heat to dissolve

pH 7.3 (orthophosphoric acid)

Sucrose (25%-1L)

250g sucrose

1L Prewash buffer

pH 7.3

Antifreeze Solution (800ml)

4.36g sodium phosphate (dibasic)

1.256g sodium phosphate (monobasic)

Dissolve fully in 320ml distilled water, then add:

240ml ethylene glycol

240ml Glycerol

All stored at 4^{o}C

Histology and Immunohistochemistry

4X Tris buffered saline (4X TBS-2L)

96g Trizma base
72g sodium chloride
2L distilled water (total)
pH 7.4

1X Tris buffered saline (1X TBS -2L)

500ml 4X TBS
1500ml distilled water
pH 7.4

0.2% Triton X-100 in TBS (TXTBS)

0.5ml Triton X-100
250ml TBS
pH 7.4

Tris non-saline (TNS)

6g Trizma base
1L distilled water
pH 7.4

Endogenous Peroxidase Quench

10ml methanol
10ml hydrogen peroxide
40ml distilled water

0.01% azide in TBS

500ml 1X TBS
2.5ml 2% sodium azide

Cresyl violet working solution

7g Cresyl violet
5g sodium acetate (anhydrous)
600ml distilled water
pH 3.5 (glacial acetic acid)
Make up to final volume of 1L

Acid alcohol

5ml glacial acetic acid
200ml 95% alcohol

Cresyl violet staining protocol

Using Shandon processing machine

70% alcohol	5 mins
95% alcohol	5 mins
100% alcohol	5 mins
50/50 chloroform/alcohol	20 mins
95% alcohol	5 mins
70% alcohol	5 mins
Distilled water	5 mins
Cresyl violet	5 mins
Distilled water	Finish

Differentiation and dehydration

On removal from the machine

70% alcohol	5 mins
95% alcohol	5 mins

Acid alcohol for destain if necessary

95% alcohol	2 mins
100% alcohol	5 mins
Xylene	5 mins

Coverslip sections using DPX

Drugs

Cyclosporine A	*Concentration*
5ml ampoule Sandimmun	5mg/ml = 250mg total
Dissolved in 20ml sodium chloride	10mg/ml
Inject 1ml/kg	10mg/kg

Appendix 3

Gestational	Time between Ultrasound and first tablet (days)													
Age	1	2	3	4	5	6	7	8	9	10	11	12	13	14
42	<10	<10	<10	<10	<10	<10	<10	<10	<10	10	11	12	12.5	13
43	<10	<10	<10	<10	<10	<10	<10	<10	10	11	12	12.5	13	14
44	<10	<10	<10	<10	<10	<10	<10	10	11	12	12.5	13	14	15
45	<10	<10	<10	<10	<10	<10	10	11	12	12.5	13	14	15	15.5
46	<10	<10	<10	<10	<10	10	11	12	12.5	13	14	15	15.5	16
47	<10	<10	<10	<10	10	11	12	12.5	13	14	15	15.5	16	17
48	<10	<10	<10	10	11	12	12.5	13	14	15	15.5	16	17	17.5
49	<10	<10	10	11	12	12.5	13	14	15	15.5	16	17	17.5	18.5
50	<10	10	11	12	12.5	13	14	15	15.5	16	17	17.5	18.5	19.5
51	10	11	12	12.5	13	14	15	15.5	16	17	17.5	18.5	19.5	21
52	11	12	12.5	13	14	15	15.5	16	17	17.5	18.5	19.5	21	22.5
53	12	12.5	13	14	15	15.5	16	17	17.5	18.5	19.5	21	22.5	24
54	12.5	13	14	15	15.5	16	17	17.5	18.5	19.5	21	22.5	24	25.5
55	13	14	15	15.5	16	17	17.5	18.5	19.5	21	22.5	24	25.5	27
56	14	15	15.5	16	17	17.5	18.5	19.5	21	22.5	24	25.5	27	28.5
57	15	15.5	16	17	17.5	18.5	19.5	21	22.5	24	25.5	27	28.5	30
58	15.5	16	17	17.5	18.5	19.5	21	22.5	24	25.5	27	28.5	30	32
59	16	17	17.5	18.5	19.5	21	22.5	24	25.5	27	28.5	30	32	34
60	17	17.5	18.5	19.5	21	22.5	24	25.5	27	28.5	30	32	34	36
61	17.5	18.5	19.5	21	22.5	24	25.5	27	28.5	30	32	34	36	38
62	18.5	19.5	21	22.5	24	25.5	27	28.5	30	32	34	36	38	40
63	19.5	21	22.5	24	25.5	27	28.5	30	32	34	36	38	40	>40
64	21	22.5	24	25.5	27	28.5	30	32	34	36	38	40	>40	>40
65	22.5	24	25.5	27	28.5	30	32	34	36	38	40	>40	>40	>40
66	24	25.5	27	28.5	30	32	34	36	38	40	>40	>40	>40	>40
67	25.5	27	28.5	30	32	34	36	38	40	>40	>40	>40	>40	>40
68	27	28.5	30	32	34	36	38	40	>40	>40	>40	>40	>40	>40
69	28.5	30	32	34	36	38	40	>40	>40	>40	>40	>40	>40	>40
70	30	32	34	36	38	40	>40	>40	>40	>40	>40	>40	>40	>40
71	32	34	36	38	40	>40	>40	>40	>40	>40	>40	>40	>40	>40
72	34	36	38	40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40
73	36	38	40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40
74	38	40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40
75	40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40
76	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40

Cardiff Foetal Tissue Bank (CFTB) standard operating procedure for staging of embryos. Showing expected CRL in mm calculated from gestational age on day of ultrasound (left), and days between ultrasound and 1st tablet (top). The white area indicates predicted CRL between 10-40mm which may be considered for clinical studies. (CFTB 2012)

Appendix 4

Antibodies

Primary antibody	Type	Supplier	Concentration	Blocking serum	Secondary Antibody (all at 1:200)
CD4 (Mouse) 550278 (Mouse $CD4^+$ helper T cells)	Rat monoclonal	BD Pharmingen	1:500 (IH)	Goat	Rabbit anti-rat
CD4 (Rat) Ab8167 (Rat $CD4^+$ helper T cells)	Mouse monoclonal	Abcam	1:500 (IH)	Horse	Horse anti-mouse
CD8 (Mouse) ab22378 (Mouse $CD8^+$ cytotoxic T cells)	Rat monoclonal	Abcam	1:1000 (IH)	Goat (Invitrogen)	Rabbit anti-Rat (BA-4000 Vector)
CD8 (Rat) MCA48GA (Rat $CD8^+$ cytotoxic T cells)	Mouse monoclonal	Serotec	1:500 (IH)	Horse	Horse anti-mouse
CD11b (Rat) MCA275G (Rat macrophages and microglia)	Mouse monoclonal	Serotec	1:1000 (IH)	Horse	Horse anti-mouse
DARPP-32	Mouse	Cornell University	1:10000	Horse	Horse anti-mouse
F4/80 ab90247 (Mouse macrophages)	Rat monoclonal	Abcam	1:1000	Goat	Rabbit anti-rat
HuNu MAB1281 (Human nuclei)	Mouse monoclonal	Millipore (Chemicon)	1:1000 (IH)	Horse (Invitrogen)	Horse anti-mouse (BA-2001 Vector)
Iba1 0919-19741 (Mouse macrophage/microglia)	Rabbit polyclonal	Wako	1:8000	Goat	Goat anti-rabbit (BA-1000)

Appendix 5

Problems with storage and degradation of tissue sections

In the Brain Repair Group established protocols are used for the processing and sectioning of brain tissue and subsequent storage, histological and immunohistochemical staining. As such, shared solutions are used which are made up by all users and technicians and stored and restocked where required to prevent wastage. The protocol for processing and storing brain tissue sections is outlined below. This was the protocol established for use by all users at the time of undertaking this PhD.

Perfusion and sectioning

- 50-100ml Prewash to flush all the vessels
- 200-250ml 1.5% PFA delivered over 5 minutes with a peristaltic pump
- Removal of brain and post-fix overnight (24hr) in 1.5% PFA
- Transfer to sucrose for storage until sectioning at 40μm

All animals (rats and mice) are transcardially perfused with 1.5% PFA rather than 4% as it was found that for a number of antibodies tested, 1.5% PFA improved full thickness staining, enabling clearer quantification of positively stained cells (Torres *et al.* 2006).

Storage

Fixation at 1.5% was considered to be adequate for tissue sections cut and stored in 96 well plates in TBZ at 4^{o}C (1 brain per plate). Sections can be stored long term in these conditions, provided levels of TBZ are monitored and topped up as required. TBZ is therefore prepared by all users when necessary and used as a shared solution.

Problems

Although these methods have been successfully implemented in this lab group for a considerable amount of time with no problems experienced, for unclear reasons I encountered severe deterioration of tissue sections from brains across a number of my experiments, as shown in **Table 5.1**. This has limited the histological assessment possible for these experiments, and therefore a number of experiments described in this thesis are lacking data. However, all experiments which showed interesting data and for which further staining could not be carried out have been repeated where possible and are quantified to the best level within the timeframe available. More extensive quantification of interesting data can be carried out prior to publication.

There are a number of potential causes for these problems; however it is most likely that a combination of these contributed to the degradation of tissue sections:

- **Fixation**

It is possible that fixation with 1.5% PFA was insufficient for storage of tissue sections at 4^{o}C. However this cannot account for why some sections from each experiment remained fine in storage long term when processed in exactly the same way. Batches of brains wrapped and stored together were affected while other batches cut on the same day and stored in the same fridge were not affected. Additionally some sections degraded after some time in storage, whereas others began to deteriorate almost immediately, making them unusable by 1-2 weeks.

- **Storage (TBZ)**

The recipe for TBZ can be found in Appendix 2. The basic solution is made up as 0.01% azide in TBS. The azide in this solution should preserve tissue sections and prevent the growth of bacteria. As the tissue sections which were damaged were processed either over a period of a couple of months, or storage plates may have been topped up over this time period, it may be that the solution used was made up incorrectly or the water was not clean enough meaning stored sections became contaminated in the fridge.

Contamination

If either of the above two points occurred, then tissue sections would either be underfixed, or inadequately protected from contamination by bacteria during storage at 4^{o}C. This is possible since initial problems were observed in stacks of plates wrapped together, but not necessarily those around it. Not all sections stored in these fridges were affected; however had these been better fixed or stored effectively in azide, such contamination would not be a problem.

Solution

Where sections had begun to degrade attempts were made to fix prior to staining in 4% PFA, and carry out required stains as quickly as possible to try not to lose any data. However although antibody staining was successful in some cases, it was impossible to mount sections onto slides as they would disintegrate.

For subsequent experiments, tissue sections were therefore stored at -20^{o}C in Antifreeze in 48 well plates (4 brains per plate). Additionally, for the most recent experiment (Chapter 7), 4% PFA was used rather than 1.5%. No detrimental effect was observed on the antibody stains in these sections, and no difficulty was experienced in the quantification of positive staining. After these changes were made no further problems were experienced and tissue sections have been successfully stored long term at -20^{o}C.

Chapter	Experiment	Fixation/Storage	Staining completed	Staining not possible
3	Experiment i)	1.5%/4^{o}C	CV, HuNu OX42 and CD8: complete but some damaged CD4: Very few and badly damaged	Staining incomplete for some immune markers
5	Experiment *i)*	1.5%/4^{o}C	CV, HuNu	NeuN, immune markers
5	Experiment *ii)*	1.5%/4^{o}C	CV, HuNu	NeuN, immune markers
5	Experiment *iii)*	1.5%/4^{o}C	CV HuNu: Not for all animals CD8: Only for a few animals	Complete HuNu Immune markers

Table 5.1 Experiments affected by degradation of tissue sections

Appendix 6

Proliferation of hPF cortical tissue following transplantation

Surviving hCTX transplants in rat hosts were consistently large at 12 weeks after transplantation as identified by both CV and HuNu staining. To determine the phenotype of surviving grafted cells; staining for NeuN was carried out on sections from **Chapter 4**, which revealed the presence of mature neurons closer to the periphery of grafts, with far less towards the centre despite confirmation of the presence of human cells with HuNu antibody staining (**Figure 1** A-C). To investigate this further, staining for Ki67 was conducted, revealing the presence of cells closer to the centre of the graft which were still proliferating at 12 weeks post-transplantation (**Figure 1**; D,E). It is not clear whether the proliferative nature of this tissue is related to the effectiveness of hCTX as a source of cells for desensitisation. The presence of a viable, proliferative population of donor cells during immune system development may be more beneficial than an alternative tissue source. It is possible that differences such as these between donor tissues may have an effect on the host immune response to cells following transplantation. I aim to further investigate both of these factors by beginning to characterise the donor tissue used to desensitise and transplant and correlate this with the success of desensitisation and the survival/rejection of transplanted tissue.

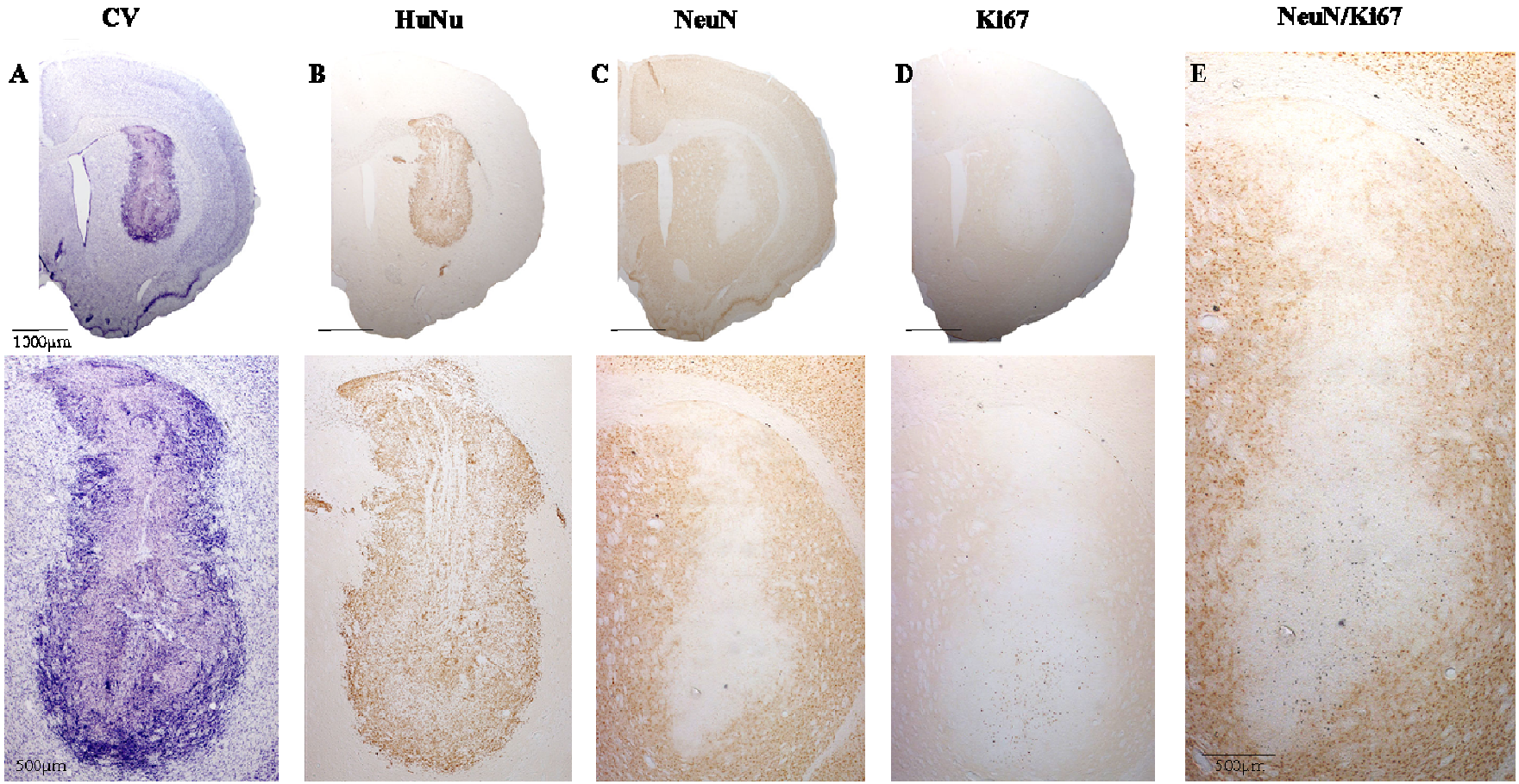


Figure 1 Photomicrographs of a hCTX graft 12 weeks after transplantation in a host desensitised with hCTX tissue from **Chapter 4** stained with CV (A), HuNu (B), NeuN (C), Ki67 (D), and NeuN and Ki67 merged (E). Images were manipulated in Adobe Photoshop in C-E to increase contrast and enhance visualisation of staining. E shows staining from subsequent sections in C and D overlaid to highlight the presence of immature proliferating cells in the centre of the graft and mature neurons around the periphery. This is typical of large hCTX grafts at 12 weeks from **Chapter 3** and **4**.

Appendix 7

Desensitisation in C57BL/6J mice

Experimental Design

A cohort of C57BL/6J (BL/6) mice (n=13) were desensitised neonatally (P0-2) with hCTX ($1x10^5$ cells) from two embryonic donors as described in **Chapter 2**. All mice received QA lesions to the right striatum followed by a transplant of hCTX ($5x10^5$ cells) 8 days later. Survival of transplants was assessed 6 weeks after transplantation and compared to a small control group (n=3) of untreated mice who received the same lesions and transplants.

Results and Conclusions

Following perfusion (1.5% PFA) and collection of brains as described previously, sections were cut at 40μm and, due to previous problems with degradation of tissue sections (Described in Appendix 5), stored at -20^{o}C. Sections were stained with CV to identify surviving grafts, and with HuNu to confirm this. As in previous experiments, survival of human xenografts transplanted to the striatum of desensitised animals was found to be poor, with no surviving grafts identified. This experiment was conducted concurrently with those described in **Chapter 5**; thus sections from desensitised BL/6 hosts were used to test and improve antibody staining. As described in **Chapter 5**, in the majority of cases where CV staining was suggestive of good transplant survival, no staining could be found in HuNu stained sections. It was reasoned that if transplant survival could not be confirmed with this human specific antibody, then it may be possible to determine whether this staining represents a transplant by showing the inverse; that this dense staining is not due to the infiltration of immune cells.

Sections from desensitised BL/6 hosts were therefore stained with a range of antibodies (described in below). Staining with these antibodies suggests that there is poor survival of transplanted cells, since little positive staining for NeuN was observed in the grafted area. Staining with Iba1 shows that the dense staining

observed in CV sections is potentially indicative of large microglial infiltration and rejection of the graft (**Figure 7.1**; A,E,I,C,G,K). Initial staining for cytotoxic (CD8+) and helper (CD4+) T cells did not show a large number of positive cells in the grafted area. To ensure this was not due to poor optimisation of the antibody in mouse tissue, a pan T cell marker (CD3) was subsequently used. This showed a large number of positive cells in the vicinity of the graft site, as well as additional positive cells distributed around the brain (**Figure 7.1**; B,F,J,D,H,L).

Although this experiment did not provide any positive data in determining whether desensitisation of BL/6 mice to hPF tissue can be achieved, findings were useful in determining the outcomes of experiments described in **Chapter 5.** The optimisation of antibody stains could be carried out to allow the identification of a strong immune response to xenogeneic human tissue transplants and highlighting that the presence of CV staining resembling surviving transplants is more likely to be due to the presence of these immune cells. A strong microglial response was observed in sections from both desensitised and untreated hosts, with the morphology of cells resembling a more activated microglial phenotype, as shown in **Figure 7.2**. This has also aided in the design of future experiments to further characterise this and with the aim of targeting immune suppression more appropriately.

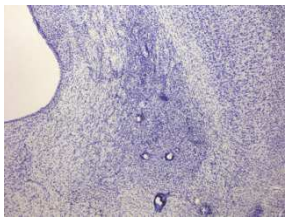
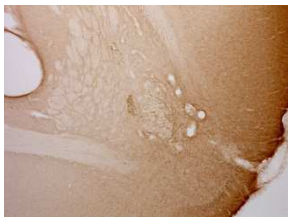
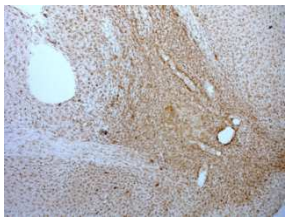
Antibody	Aim	Finding	
CV: Nissl body stain	To identify graft region	Dense staining on some sections	
NeuN: Mature neuronal marker	To detect neurons in the transplant area and see if this corresponds to CV staining	The area identified as a graft in CV staining shows no staining with NeuN	
CD4: $CD4^+$ Helper T cells		Some positive staining for both T cell markers but insufficient to account for density of CV	
CD8: CD8+ Cytotoxic T cells	To identify whether CV staining is due to dense lymphocyte infiltration		
CD3 : Pan T cell marker		Large amounts of positive staining in the grafted area and distributed around the brain	
F4/80: Mature mouse macrophages and microglia	To identify whether CV staining is due to an increased microglial response to transplant	Increased staining of microglia but insufficient to account for CV staining	
Iba1: Microglia and macrophages		Dense staining in grafted area corresponding to CV staining	

Table 7.1 Antibodies tested to identify source of dense CV staining

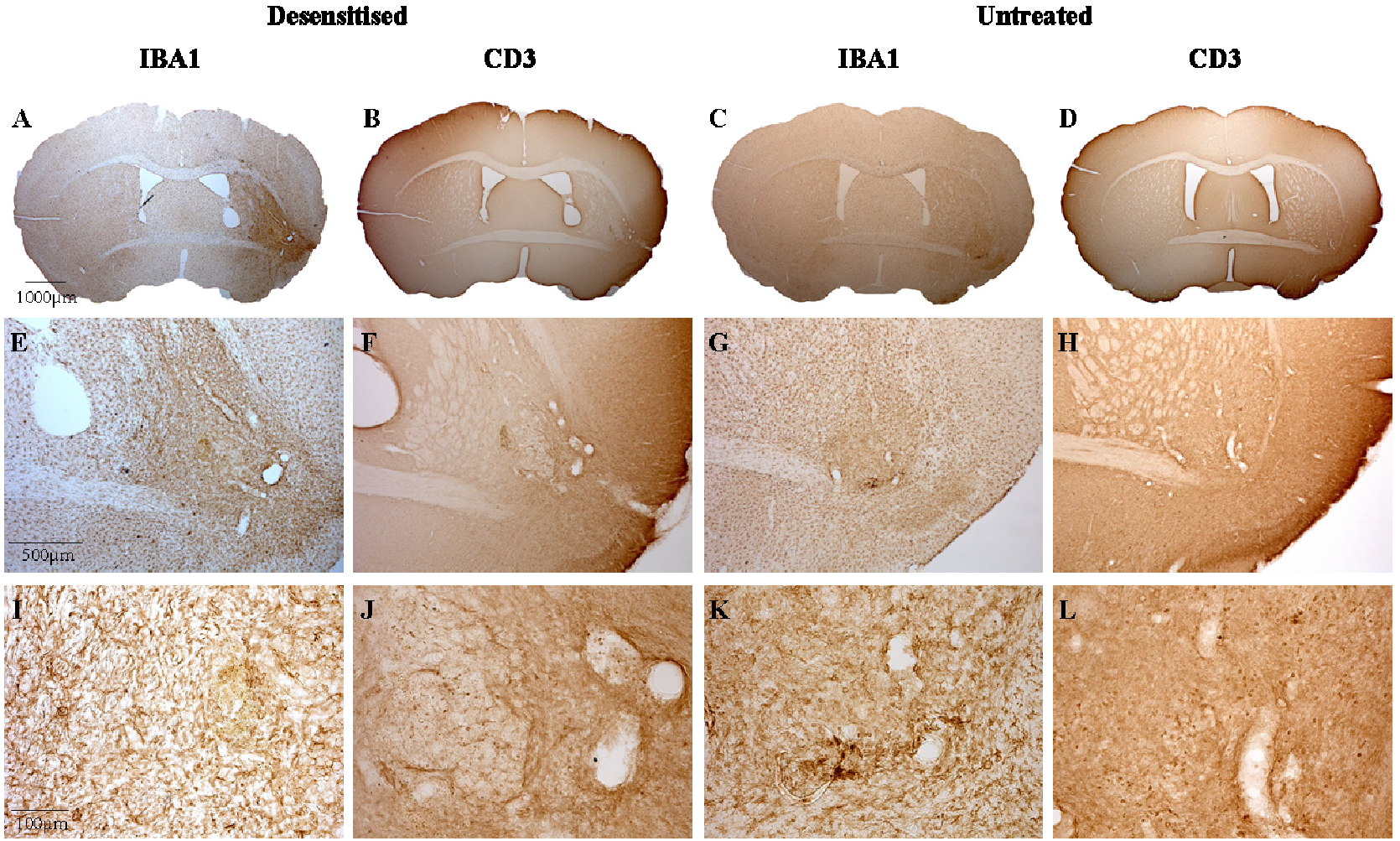


Figure 7.1 Photomicrographs of sections from BL/6 mouse hosts transplanted with hCTX which had previously been desensitised (left panels) with hCTX tissue or were untreated (right panels). Sections are stained with the microglial marker Iba1 (A,E,I; C,G,K) and pan T cell parker CD3 (B,F,J; D,H,L). Intense staining for both microglia and T cell immunoreactivity was seen in all hosts.

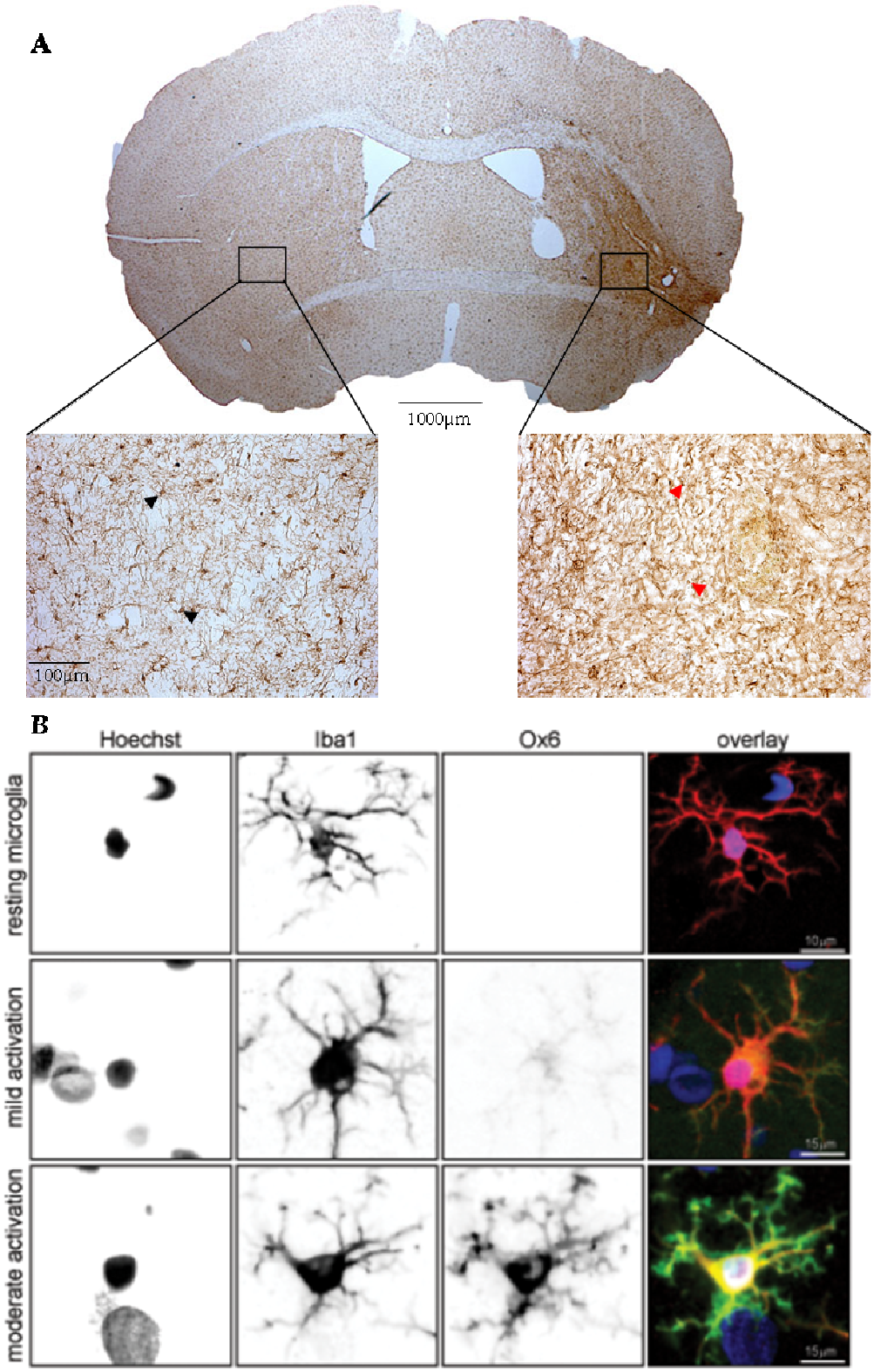


Figure 7.2 Iba1 immunoreactivity. A) Microglia in a mouse host transplanted with hCTX, showing resting, ramified microglia in the left striatum (black arrowheads) and activated microglia in the grafted area of the right striatum. B) Examples of the morphology of resting and activated microglia (from (VanGuilder *et al.* 2011))

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