PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B

Phil. Trans. R. Soc. B (2008) **363**, 153–170
doi:10.1098/rstb.2006.2018
Published online 1 March 2007

Neurodegeneration and cell replacement

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The past decade has witnessed ground-breaking advances in human stem cell biology with scientists validating adult neurogenesis and establishing methods to isolate and propagate stem cell populations suitable for transplantation. These advances have forged promising strategies against human neurodegenerative diseases. For example, growth factor administration could stimulate intrinsic repair from endogenous neural stem cells, and cultured stem cells engineered into biopumps could be transplanted to deliver neuroprotective or restorative agents. Stem cells could also be transplanted to generate new neural elements that augment and potentially replace degenerating central nervous system (CNS) circuitry. Early efforts in neural tissue transplantation have shown that these strategies can improve functional outcome, but the ultimate success of clinical stem cell-based strategies will depend on detailed understanding of stem cell biology in the degenerating brain and detailed evaluation of their functional efficacy and safety in preclinical animal models.

Keywords: human embryonic stem cells; foetal stem cells; adult neural stem cells; neurodegenerative disease; immunology

1. INTRODUCTION

The past decade has witnessed ground-breaking advances in human stem cell biology. Multipotent neural stem/progenitor cells, harvested from foetal and adult human brains, have been propagated as genetically stable cell lines (Svendsen *et al.* 1998; Carpenter *et al.* 1999; Vescovi *et al.* 1999). In addition, the pluripotent stem cells have been isolated from human blastocysts and expanded indefinitely as stable cell lines (Thomson *et al.* 1998; Amit *et al.* 2000; Reubinoff *et al.* 2000). The generation of human embryonic, foetal and adult stem cell lines is exciting because these cell sources could contribute different but, perhaps, equally important properties to strategies against human neurodegenerative diseases.

These stem cell advances have been paralleled by increasing demand for effective neurodegenerative disease treatment in a rapidly aging world population. In the United States alone, four million patients suffer from Alzheimer's disease (AD), one million from Parkinson's disease (PD), 350 000 from multiple sclerosis (MS) and 20 000 from amyotrophic lateral sclerosis (ALS). Worldwide, these incurable neurodegenerative diseases produce immeasurable societal strain as they afflict more than 20 million people.

Each neurodegenerative disease requires a tailored approach to treat its unique pathology and progression. Diseases like PD, Huntington's disease (HD) and ALS affect specific brain regions or cell types. Others like MS or AD are more diffuse, and 're-wiring' strategies would be difficult at best. These 'diffuse' diseases may be more amenable to neuroprotective strategies using cell-based delivery of traditional drugs or neuroprotective growth factors.

Three fundamentally different stem cell strategies could lead to a cure for neurodegenerative disease. Stem cells could deliver bioactive proteins or peptides to modify or biomodulate the disease process. At the opposite end of the spectrum, stem cells could generate exact neuronal or glial cells to replace cells lost in disease-affected systems. These cells could be oligodendrocytes for remyelination in MS or neurons for restoring affected circuit function in PD. Although fully restoring a degenerated neural network is a worthy, yet lofty, goal, disease symptoms could be alleviated by introducing stem cell-derived neurons ectopically to modulate an affected network (figure 2).

2. THE STEM CELL PLAYERS

Stem cells from different sources have unique attributes that will differentially affect their suitability for use in therapeutic strategies. For example, stem cell proliferative capacity is negatively correlated with donor age. Human embryonic stem cells (hESCs) divide at least 300 times (every 36–120 h) and are often described as virtually immortal (figures 1 and 2). Foetal neural stem cells divide up to 100 times (every 3–4 days) in culture (still yielding very substantial total cell numbers) and adult neural stem cells divide very few times *in vitro* (Svendsen *et al.* 1996, 1998; Vescovi *et al.* 1999; Arsenijevic *et al.* 2001; Richards *et al.* 2002; Cowan *et al.* 2004). The limited proliferative capacity of adult neural stem cells precludes their use in most clinical

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One contribution of 14 to a Theme Issue 'Stem cells and brain repair'.

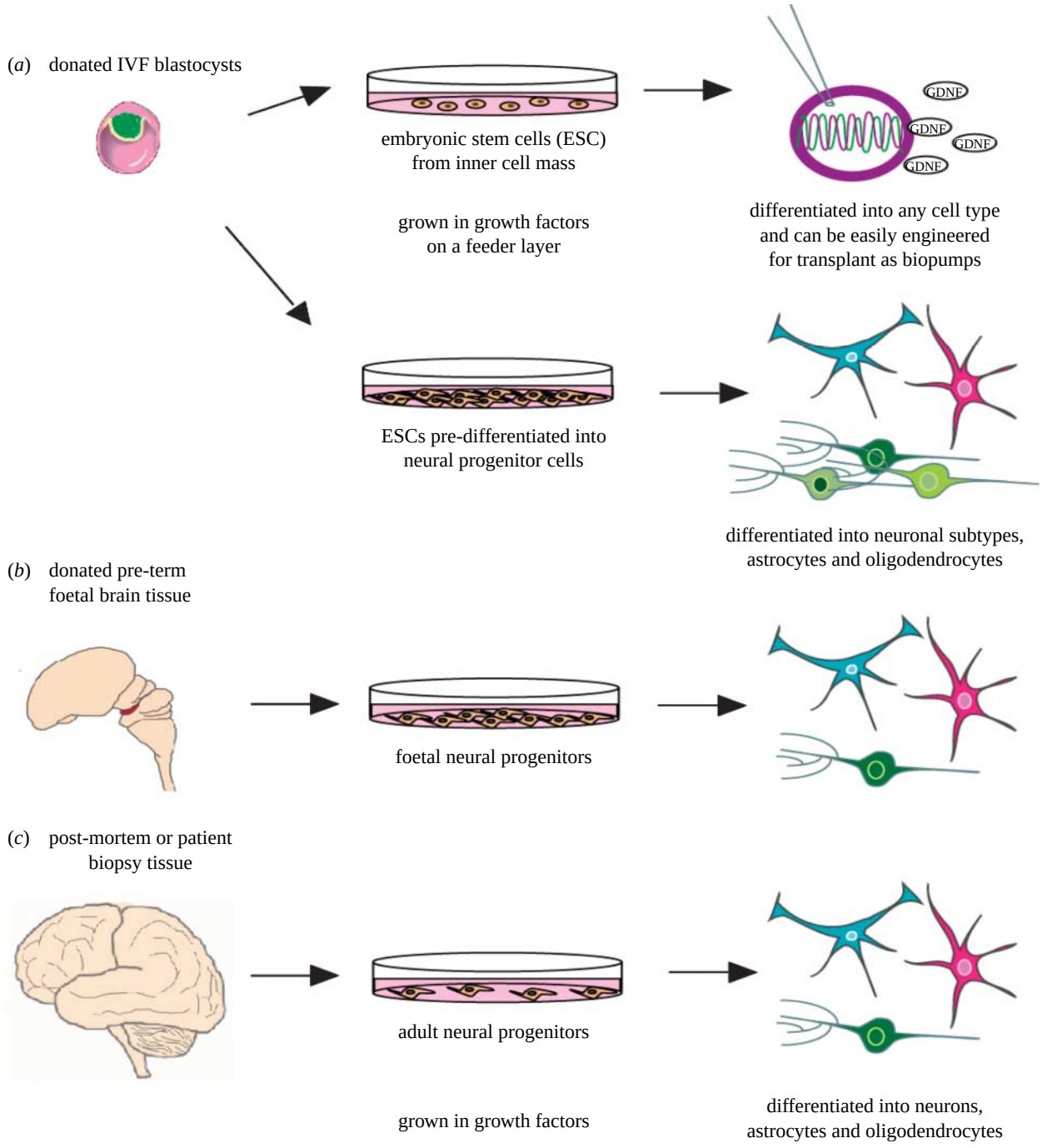

Figure 1. Human stem cells grown from different tissue sources have properties that each may be advantageous for different therapeutic strategies. (*a*) Human embryonic stem cells (hESCs) derived from excess blastocysts that have been donated by women who have undergone *in vitro* fertilization (IVF) generate millions of cells with unlimited potential (they can generate all cells in the body). In their primitive form, hESCs can be easily engineered to secrete neurotransmitter or growth factors to stop disease progression or to augment intact circuitry, respectively. When hESCs are differentiated into neural progenitors, they can then make neurons, astrocytes and oligodendroctyes, the precursors of which could potentially be used to regenerate dying circuitry. (*b*) Foetal neural progenitors harvested from the brain tissue of discarded foetuses can be expanded for use in thousands of patients. They readily generate neurons and astrocytes *in vitro* and generate neurons when transplanted into neurogenic areas of the adult brain. (*c*) Adult neural progenitors can be harvested from post-mortem tissue or even from patient biopsies to generate immunologically matched transplants. These cells generate neurons, astrocytes and oligodendrocyte precursors in culture and neurons when transplanted into neurogenic regions of the adult brain. The biggest limitation with adult neural progenitors is that they rapidly senesce in culture preventing large-scale expansion.

applications. hESCs, the most immature stem cells, exhibit the highest potential to generate various neuron types relevant for degenerative disease, such as dopaminergic neurons for PD (Ben-Hur *et al.* 2004; Perrier *et al.* 2004; Park *et al.* 2005) or motor neurons for ALS (Li *et al.* 2005). Neither foetal nor adult neural stem cells appear to produce either cell types in significant abundance (Caldwell & Svendsen 1998; Dziewczapolski *et al.* 2003). However, both foetal and adult neural stem cells readily generate glia, which could be engineered to carry and express genes that produce neuroprotective agents.

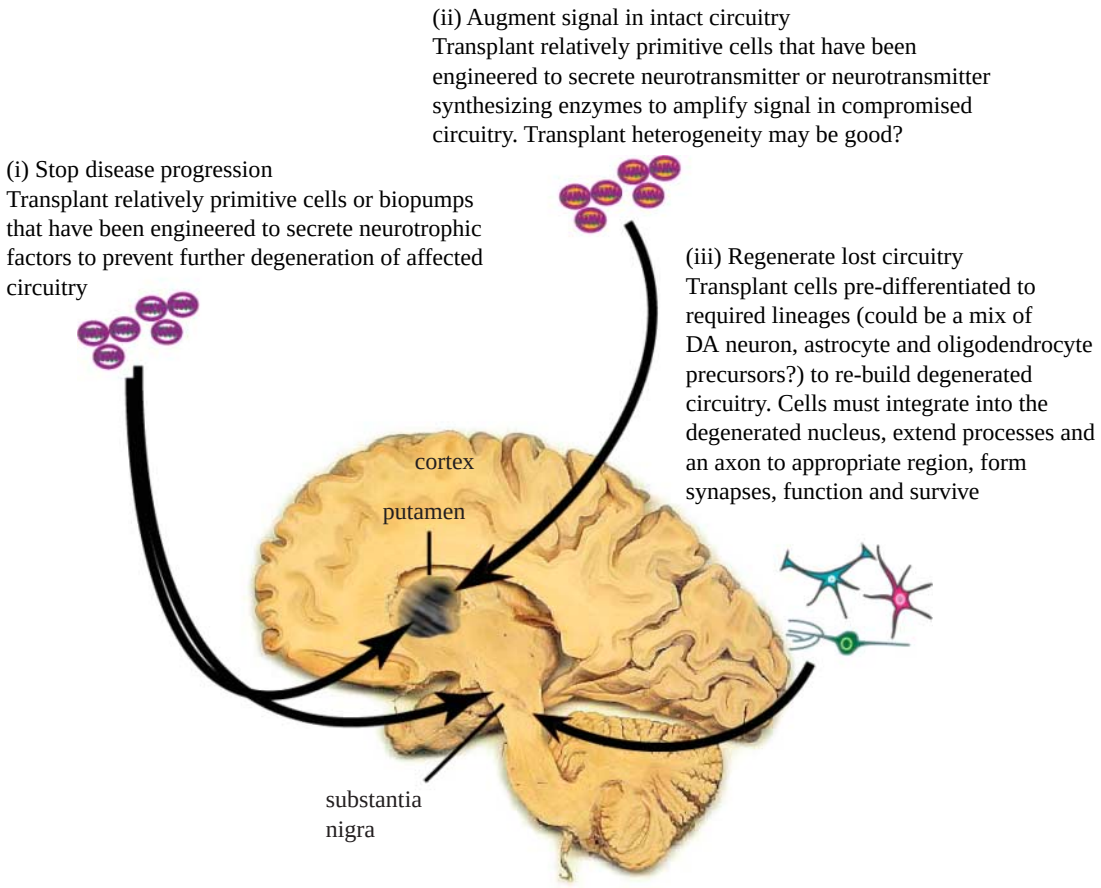

Figure 2. Stem cell strategies for treating neurodegenerative diseases. We propose that a multifaceted stem cell transplant approach would provide the most significant benefit for patients suffering from neurodegenerative disease. In the case of Parkinson's disease, halting the degeneration of substantia pars compacta (SNpc) neurons could prevent further degradation of motor ability. Augmenting the signal in remaining circuitry could improve the motor symptoms associated with the disease. Finally, regenerating lost circuitry could abolish disease symptomology.

Human embryonic stem cells (hESCs) are derived from the blastocyst inner cell mass of excess embryos generated by *in vitro* fertilization. *In vitro*, hESCs maintain a normal karyotype for extended passages while generating copies of themselves and any cell type found within the three germ layers (except trophoblasts) upon directed differentiation, providing an unlimited source of cells for central nervous system (CNS) transplantation (Hoffman & Carpenter 2005). hESCs directed into neural precursors can generate neurons, oligodendrocytes and glia, both in culture and when transplanted into the adult rodent CNS (Reubinoff *et al.* 2001; Zhang *et al.* 2001; Keirstead *et al.* 2005; Tabar *et al.* 2005). The ease with which hESCs can be expanded in culture as normal diploid cells is a significant advantage over foetal and adult stem cells that require immortalization via mutation or proto-oncogene introduction to escape eventual senescence. Although useful for expansion, proto-oncogene expression introduces tumour formation risk. On the other hand, undifferentiated hESCs potently generate teratomas following transplant. Thus, one challenge for hESC strategies will be to ensure full differentiation prior to transplant and/or the ability to engineer them for selective death post-transplant, if required.

Foetal neural stem cells harvested from the post-mortem human foetal brain maintain a normal karyotype for a significant number of passages in culture and can produce a large number of neurons and astrocytes both *in vitro* and in neurogenic regions of the adult rodent CNS following transplant (Svendsen *et al.* 1996, 1998; Fricker *et al.* 1999; Vescovi *et al.* 1999; Cummings *et al.* 2005). Their relatively high proliferative capacity permits the generation of cells for many patients from a single donor. Still, foetal lines would need to be re-derived and re-characterized frequently if they are to see extensive clinical use. One significant advantage that foetal cells hold over hESCs is that they do not appear to generate tumours following transplant.

Adult neural stem cells persist throughout life and mediate limited repair and restoration through adulthood (Gage 2003). The opportunity to encourage repair by endogenous stem cells could become significant if signals controlling their behaviour were better understood (Emsley *et al.* 2005). Adult neural stem cells can be harvested from brain tissue, post-mortem or through biopsy and expanded in culture. These cells exhibit genetic stability over many passages and differentiate into limited numbers of neurons, astrocytes and oligodendrocytes, both *in vitro* and upon transplant into the rodent CNS (Roy *et al.* 2000; Arsenijevic *et al.* 2001; Palmer *et al.* 2001; Nunes *et al.* 2003). Oligodendrocyte progenitor cells harvested from adult white matter have even remyelinated the newborn shiverer mouse brain upon transplant (Windrem *et al.* 2004). If the proliferative capacity

and neurogenic potential of these cells could be significantly enhanced, then a clear advantage of their use would lie in the ability to generate autologous cell transplants from small biopsies of neural tissue.

Non-neural adult stem cell alternatives to those derived from the brain tissues have been proposed for treating CNS diseases. For example, mesenchymal lineage cells are easily harvested from bone marrow, skin or adipose tissue as are haematopoietic lineage cells from blood, theoretically providing sources of transplantable autologous neurons or glia for CNS therapy (Joannides *et al.* 2004; Ortiz-Gonzalez *et al.* 2004; Crain *et al.* 2005; Kokai *et al.* 2005; Toma *et al.* 2005). Although haematopoietic stem cells, for example, do acquire some phenotypic properties of neurons and glia upon transplant to the rodent CNS, they retain haematopoietic cell properties (Massengale *et al.* 2005) and may not function as authentic CNS cell types. This property may preclude non-neural-derived stem cells as candidates for reconstruction, but could be extremely valuable for producing autologous biological delivery vehicles for neuroprotective gene products.

A single stem cell type is not ideal for all applications. Cultured hESCs grow very efficiently and maintain pluripotency, but cancer risk remains a significant deterrent to their clinical use. Foetal brain-derived neural stem cells are not tumorigenic, but there are ethical and logistical problems in sourcing tissues for all individuals that might benefit from their therapeutic use. Adult stem cells are an attractive source of cells for generating autologous cell transplants, but are not practical because they rapidly senesce in culture. Non-neural cells, such as mesenchymal stem cells, can be easily isolated from patients to produce autologous cell transplants, but we do not yet know how to convert them into authentic CNS cells, which is the ultimate goal of restorative therapies.

3. THE PRINCIPAL DISEASE CHALLENGES

Alzheimers disease (AD), the most common sporadic neurodegenerative disease, histologically consists of accumulated neurofibrillary tangles, β-amyloid aggregates and pyramidal neuron degeneration, most prominently in the neocortex, hippocampus and amygdala. AD symptoms include progressively impaired general cognitive function that culminates in the inability to care for oneself and death within 3–20 years (Mann 1996). The broad multisystem neuronal loss in AD is particularly problematic for neural restoration using transplants, but could benefit from protective strategies and/or strategies that stimulate endogenous stem cell repair.

Parkinson's disease (PD) pathology initially involves the highly selective substantia nigra pars compacta (SNc) dopamine neuron death. These neurons project to the striatum where motor function is regulated. This pathology is accompanied by intracellular protein aggregation leading to 'Lewy body' inclusions (Iacopino & Christakos 1990; Yamada *et al.* 1990; Ito *et al.* 1992). Motor symptoms that include tremor, muscle stiffness, paucity of voluntary movements and postural instability develop as dopamine neurons die depleting the striatal dopamine levels. Dopamine depletion can be reversed in early disease stages by simply increasing the systemic dopamine levels with appropriate drugs. The motor symptoms can also be reduced by foetal tissue-derived dopamine-producing cells transplanted into the caudate nucleus of the striatum (Lindvall & Bjorklund 2004). Promising outcomes are being generated by the studies testing the stem cell-derived dopamine neuron transplants in preclinical models (Sanchez-Pernaute *et al.* 2005). Although PD pathology is focal initially, repair becomes increasingly challenging in later stages when multiple dopamine systems degenerate to produce numerous non-motor symptoms. Thus, strategies attempted early in the disease process will probably be most effective.

Multiple sclerosis (MS), the most common demyelinating disease of the CNS, is a chronic recurring autoimmune assault on oligodendrocytes that ultimately produces patchy demyelination with subsequent axonal loss and reactive gliosis (Lassmann *et al.* 2001). The immune lesions are initially repaired by endogenous precursors that generate remyelinating oligodendrocytes, but remyelination fails after repeated immune system attacks (Franklin 2002). These lesions produce variable symptoms including impaired vision and declining coordination, sensation, speech, swallowing, bladder control and cognitive function, as well as weakness, fatigue and diminished sexuality (Mitchell *et al.* 2005). Successful stem cell-mediated repair would clearly introduce myelinating oligodendrocytes along with an effective strategy to prevent further autoimmune insult (see Chandran *et al.* 2007).

Amyotrophic lateral sclerosis (ALS) pathology consists of the progressive upper and lower cholinergic motor neuron, as well as small interneuron, degeneration. Familial ALS forms caused by superoxide dismutase gene mutation implicate mitochondrial dysfunction, but most ALS cases are sporadic with unclear aetiology. Consistent with many degenerative diseases, intraneuronal protein aggregation precedes corticospinal and corticobulbar neuron degeneration. This degeneration produces rapidly progressing muscle weakness, atrophy, spasticity and ultimately respiratory failure-induced death within a few years of disease onset (Wharton & Ince 2003). Early data indicate that hESCs can produce lower motor neurons (Li *et al.* 2005). However, motor neurons are broadly distributed and extend long-distance projections, producing unique challenges for cell-replacement strategies. Therefore, neuron-protective strategies may be more imminently realistic for ALS treatment.

Huntington's disease (HD) is an autosomal dominant disease involving mutations in the *huntingtin* gene. Medium spiny gamma aminobutyric acid-ergic (GABAergic) interneurons of the striatum and neocortex atrophy and die, leading to a choreic movement disorder, progressive dementia and death typically within 15–20 years (Dunnett & Rosser 2004). Here, the cell type is quite specific, the aetiology known and the affected brain regions are relatively discrete. Because many neural stem cell populations seem to be able to generate GABAergic interneuron-like populations, they may be particularly effective in restoring striatal function through cell replacement (Caldwell *et al.* 2001; Jain *et al.* 2003).

Inborn errors of metabolism, the lowest hanging fruit? CNS lysosomal and peroxisomal storage disorders are produced by inherited gene defects in lysosomal enzymes. In Hurler's syndrome and Krabbe's disease, the defective enzyme is associated with an accumulation of the enzyme's normal substrate which becomes neurotoxic and ultimately results in death (Krivit 2004). Hurler's syndrome, which involves a deficiency in α-l-iduronidase, results in the accumulation of glycosaminoglycans (GAGs) and global neuronal toxicity (Staba *et al.* 2004). Krabbe's disease involves the accumulation of galactocerebroside due to the defects in the enzyme galactocerebrosidase. This interferes with myelination in the CNS and peripheral nervous system (Escolar *et al.* 2005).

Children are born with normal neurological function, but accumulation of mucopolysaccharides produces early CNS deterioration causing peripheral defects such as cardiac abnormalities, skeletal abnormalities, corneal clouding and hepatomegaly. Death occurs in early childhood (Neufeld & Munzer 2001; Wenger *et al.* 2001). Because mucopolysaccharides are trafficked intercellularly, enzyme replacement in some cells can adequately reduce GAG accumulation in many cells. In some cases, a procedure as well established as bone marrow transplants from a normal donor can restore enzyme activity by lowering GAG burden in brain cells. Successful engraftment improves overall survival, pathology and neurological function (Peters *et al.* 1996; Krivit *et al.* 1998). Krabbe's children treated before they become symptomatic remain asymptomatic through childhood. On the other hand, infants who have become symptomatic have minimal neurological improvement, suggesting that restoration of enzyme function does not reverse the damage already done (Staba *et al.* 2004). In these cases, neural repair might also be required, but the neurological injury is so diffuse that it is difficult to rationally approach the problem.

4. THE STRATEGIES

In all diseases where cell loss proceeds to the point of functional impairment, protecting the remaining neurons as well as augmenting the output of lost circuitry will become important. Neuronal protection could be drug-based or mediated via neuroprotective cell product delivery using cellular transplants. Circuit 'augmentation' would ideally be mediated by cell transplants that integrate to bolster function, but in some cases, circuit output might be augmented by simply providing neurotransmitter in bulk fashion (i.e. non-synaptically), similar to using oral L-DOPA to treat PD motor symptoms. Cellular delivery would constrain the effects of transmitters or transmitter precursors to the local area of the transplant, eliminating the potential complications associated with systemic delivery. The ultimate goal of the neural repair is to fully restore authentic circuitry. This goal is daunting in its potential complexity, but there are circumstances in which the brain does spontaneously mediate replacement by adding neurons to existing circuitry. Stem/progenitor cells are obvious candidates for neuroprotection, local augmentation or full restoration, since they can be expanded and differentiated in culture and successfully transplanted. In addition, they are easily engineered to express transgene products prior to transplantation and thus act as local *biopumps* (figure 2; Corti *et al.* 1999; Wu *et al.* 2002; Menendez *et al.* 2004; Schmidt *et al.* 2005).

(a) *Cellular delivery of neuroprotective agents*

Neurotrophins are a class of proteins that enhance neuronal survival, and neurotrophic factor delivery attenuates the behavioural and the neurobiological consequences of CNS damage in animal models of AD, PD, HD, ALS and MS (Blesch & Tuszynski 2004; Maier *et al.* 2004). Cellular biopump-mediated delivery of neurotrophins is attractive for many reasons. Neurotrophins are large peptide molecules that diffuse poorly through the blood–brain barrier and parenchyma, and their systemic delivery produces a plethora of negative side effects (Ebadi *et al.* 1997), making central delivery most safe and effective. Neurotrophins have been successfully administered intraparenchymally using bolus injection of purified protein, chronic pump infusion and direct delivery of transgenes encoding trophic factors via replication-deficient viral particles. These strategies have produced robust protective effects at the cannula site, but lack of diffusion within the parenchyma limits widespread efficacy (Gash *et al.* 1998; Kordower *et al.* 2000; Grondin *et al.* 2002; Gill *et al.* 2003). Here, cellular grafts that migrate within the local parenchyma and produce a steady local supply of trophic factor may be ideal.

(i) *Neurotrophins and PD*

Glial cell line-derived neurotrophic factor (GDNF) protects dopaminergic, cholinergic and GABAergic neurons from toxin-induced death and promotes fibre outgrowth when delivered intraventricularly or intraparenchymally in animal models of PD (Kirik *et al.* 2004; Eslamboli *et al.* 2005).

The success of animal models formed the backdrop for a clinical trial in which PD patients were given monthly intraventricular injections of GDNF. GDNF treatment neither improved clinical PD symptoms nor recovered striatal dopamine levels and the patients reported side effects (Kordower *et al.* 1999*b*; Nutt *et al.* 2003). Based upon the hypothesis that striatal GDNF levels were too low to produce positive clinical effects and that the widespread dispersal through the cerebrospinal fluid produced the observed side effects, Gill *et al.* (2003) initiated an open-label clinical trial in which PD patients received chronic striatal infusions of GDNF. Now in the second year of treatment, patients report no side effects (Patel *et al.* 2005) and remarkably, significantly improved off-medication motor and activities of daily living subscores on the unified PD rating scale (UPDRS) and on a quality of life measure. Medication-induced dyskinesias were significantly improved and increased striatal and nigral dopamine storage was detected by magnetic resonance imaging and positron emission tomography. This led to an open-labelled trial in which patients were implanted unilaterally with an intraputaminal catheter that infused GDNF for six months. These patients also exhibited improved UPDRS motor

scores in both the on and off state (Slevin *et al.* 2005). Although neither study contained controlled double-blind trials, these promising results demonstrate the safety of chronic central GDNF treatment for human neurodegenerative disease. More recently, Love *et al.* (2005) examined the brain of one of the five patients who participated in the Patel *et al.* study (2005) who died of myocardial infarction three months after the cessation of treatment. Neuropathological results showed a marked local increase in tyrosine hydroxylase (TH) positive nerve fibres, which could account for the enhanced [^{18}F]DOPA uptake and sustained clinical improvement. While the intraputaminal delivery of GDNF is clearly more beneficial than intracerebroventricular delivery, Behrstock & Svendsen (2004) argue that outcome of this treatment may be further improved using *ex vivo* gene therapy, i.e. stem cells engineered to secrete GDNF, which migrate throughout degenerating striatum to provide more diffuse GDNF delivery. Viral vectors engineered to permit an externally regulated transgene termination if the patient develops unwanted side effects are another valid mode of GDNF delivery (Kirik *et al.* 2004).

(ii) *Neurotrophins and ALS*

During development, both GDNF and human ciliary neurotrophic factor (hCNTF) are potent motor neuron trophic factors, promoting sprouting and survival (Richardson 1994; Klein *et al.* 2005), which are very desirable effects for ALS treatment. This observation led to early clinical trials, in which hCNTF was administered systemically to treat ALS, that were halted during phase II/III owing to severe side effects (ALS CNTF Group 1996; Miller *et al.* 1996*a*,*b*; Penn *et al.* 1997), analogous to those reported in rats treated systemically (Hagg & Varon 1993; Henderson *et al.* 1994). In contrast, no adverse effects were reported by ALS patients who were transplanted intrathecally for 17 weeks with encapsulated baby hamster kidney cells engineered to secrete hCNTF (Aebischer *et al.* 1996). Importantly, ALS symptomology progressed in that study, but the short study duration confounded assessment of whether disease progression was slowed by the treatment.

Klein *et al.* (2005) have reported very promising results in the SOD1$^{G93A+/-}$ rat model (which shows rapid degeneration of motor neurons, paralysis and respiratory arrest) using transplanted human foetal stem cells transduced with a lentiviral construct that permitted tetracycline-inducible GDNF expression. GDNF expression was maintained for the 11-week experiment without producing any adverse behavioural effects (measured using the blood–brain barrier test), and markers of cholinergic function were upregulated in the neighbouring nerve fibres. Similarly, intrathecal insulin-like growth factor-1 (IGF-1) administration improves motor performance, delays disease onset and prolongs the life of SOD1^{G93A} mice, partially by preventing motor neuron loss (Nagano *et al.* 2005). Beneficial effects can also be seen when IGF-1 is presented to the nerve terminals within the muscle itself. Here, Kaspar *et al.* (2003) use viral vectors to deliver and express IGF-1 within muscles to protect motor neurons and improve the survival of mice that express a mutant human SOD-1 gene. More recently, the same group has shown that the effect of IGF-1 and exercise synergize in these mice and argue that this combined treatment is the most promising for ALS to date (Kaspar *et al.* 2005).

(iii) *Neurotrophins and HD*

Growth factors exert potent neurotrophic effects on medium GABAergic spiny interneurons in animal models of HD (Kordower *et al.* 1999*a*; Mittoux *et al.* 2000; de Almeida *et al.* 2001).

The success of centrally delivered neurotrophin for treating HD in rodent and non-human primate models and the demonstration that encapsulated hCNTF-secreting cells are safe and well tolerated by ALS patients (Aebischer *et al.* 1996) led to clinical trials in which encapsulated baby hamster kidney cells engineered to secrete hCNTF were tested in HD patients. Bloch *et al.* (2004) transplanted encapsulated hCNTF-secreting cells into the lateral ventricles of HD patients every six months for 2 years. Patients reported no side effects and the study successfully demonstrated safety and feasibility. However, no significant neurological, neuropsychological, motor or striatal [^{18}F]fluorodeoxyglucose measure improvements were observed, which is perhaps not surprising based upon the results of clinical studies in which GDNF only provided clinical benefit for PD patients when administered intraparenchymally. The HD study conducted by Bloch *et al.* (2004) does show that long-term central CNTF production does not produce significant adverse side effects and, in association with clinical trials showing that intraparenchymal GDNF delivery is associated with clinical improvement in PD patients (Gill *et al.* 2003), opens an exciting avenue for investigating the potential of human stem cells engineered *ex vivo* to secrete neurotrophins.

(iv) *Lessons from preclinical and clinical studies*

Intraparenchymal delivery of neurotrophic factors has shown excellent promise both in preclinical PD, HD and ALS animal models and in clinical studies. Generating biopumps from human stem cells could improve clinical outcome relative to prior strategies since human stem cells could migrate to deliver neurotrophin throughout a degenerating CNS area. However, several technical aspects need to be addressed before this strategy is attempted clinically. These include the demonstration of long-term safety and efficacy in primate models and the ability to scale up cellular production for widespread clinical use.

For many biopump applications, the expansion of non-immortalized cell populations for transplant can be problematic, given the limited proliferative capacity of most human cell types. For example, adult human neural progenitor cells will replicate in culture only for a limited period of time (Palmer *et al.* 2001). Neural stem cells from foetal tissues have longer *in vitro* lifespans and are actively being considered for clinical use (Behrstock & Svendsen 2004). A better long-run solution might be the use of neural progenitor cells that are derived from hESCs. hESCs have very high proliferative capacity and are considered by many to be immortal (Cummings *et al.* 2005; Maitra *et al.* 2005). In addition, transplanted hESC neural cell

derivatives are indistinguishable from their *in vivo* counterparts (Tabar *et al.* 2005), suggesting that their functional integration could be superb. However, undifferentiated genotypically normal hESCs readily form teratomas and the cancer risk associated with transplants contaminated with undifferentiated hESCs remains the largest barrier to their clinical use.

(b) *Local systems augmentation*

Degenerating circuitry augmentation is a strategy that has been investigated for many decades in animal models of neurodegenerative disease and over the past 20 years in the clinical studies of PD and HD. In these studies, compromised neurotransmitter levels are augmented by adding a local neurotransmitter source. This can improve the symptomology associated with a given neurodegenerative disease. While not exactly recapitulating lost circuitry, bulk delivery of neurotransmitters or enzymes that catalyse neurotransmitter production locally can modulate remaining circuitry to prolong function.

Ectopic foetal neuron transplants have shown that functional outcome can be improved without exactly replacing the affected circuitry in animal models of neurodegenerative disease. For example, in both rat and non-human primate models of PD and HD, foetal tissue-derived neuroblasts transplanted into the striatum survive and reverse both motor and cognitive deficits (Freeman 1997; Kordower *et al.* 1997; Peschanski *et al.* 2004*b*). Promising preclinical model results have led to several clinical trials for PD and a few for HD (Lindvall *et al.* 1989; Kordower *et al.* 1995, 1997; Freeman 1997; Clarkson 2001; Le Belle & Svendsen 2002; Peschanski *et al.* 2004; Winkler *et al.* 2005). Recent stem cell biology advances have shown that massive numbers of dopamine neurons with midbrain attributes can be generated from hESCs. The availability of these more easily obtained and expandable dopamine neuron sources generates the exciting opportunity to reiterate the grafting successes for PD and more systematically investigate sources of grafting failures. Incorporating the lessons learned from foetal tissue transplant studies will provide extremely valuable guidance to the field as it moves forward with stem cell strategies.

(i) *Foetal tissue transplants and PD*

Lindvall *et al.* (1989) reported the results of the first human clinical trial, which transplanted human foetal tissue into the putamen of PD patients. The grafts, harvested from 8- to 10-week-old aborted human foetuses, produced modest motor improvement in two late stage PD patients. Out of the approximately 300 PD patients who have since undergone the procedure, only a few have enjoyed the long-term positive clinical benefits (Clarkson 2001). The difficulty with placebo effects in these open-label foetal tissue transplant trials led the National Institutes of Health to initiate two double-blind placebo-controlled studies in 1993 (Freed *et al.* 2001; Olanow *et al.* 2003). The double-blind procedure meant that although all patients were prepared for surgery (they were anaesthetized and a burr hole was drilled through the skull) some were not given transplants. Keeping this information from the patients and investigators prevented possible placebo effects from confounding interpretation of the trial outcome. In the Denver–Columbia trial (Freed *et al.* 2001), human ventral mesencephalic tissue pooled from two foetal brains was cultured for up to four weeks prior to bilateral transplant into the putamen of patients. No persistent improvement in the motor part of the UPDRS was observed among transplant patients at 12 months. However, in a subgroup of younger patients (below 60 years), a statistically significant 30–35% reduction was observed across the post-graft evaluation period. Post-mortem exams conducted on the brains of the two patients who died from unrelated causes after grafting revealed that the number of surviving dopaminergic neurons was half of what had been reported in open-labelled trials (Winkler *et al.* 2005). In the Tampa–Mount Sinai trial (Olanow *et al.* 2003), human ventral mesencephalic tissue from one or four donors was cultured for 2 days and then transplanted as solid pieces post-commissurally and bilaterally into each putamen. A progressive six-month post-surgical improvement in UPDRS scores was observed but gradually declined to no significant difference at 24 months.

Whether differences in methodology or placebo effects contributed to the successes observed in the open-label trials, relative to the double-blind placebo-controlled trials, is unclear. Compounding factors probably led to the failure and unexpected side effects, such as dyskinesias, observed in the placebo-controlled trials, that could include lack of immunosuppression, quality and consistency of transplanted tissue, patient selection and transplant parameters. Clearly, these crucial issues remain to be resolved and are discussed in a recent detailed review by Winkler *et al.* (2005).

(ii) *The challenge for stem cell-mediated augmentation strategies*

Several questions emerge from the foetal tissue studies that could potentially be answered using the stem cells. The exact cellular composition of an ideal transplant is largely unknown and stem cells strategies could test how controlled glia and/or neuronal subtypes numbers (graft content) influence transplant efficacy and outcome in animal models. Also, the increasing abundance and variety of hESC lines can provide choices in major histocompatibility complex (MHC) matching that have been completely unavailable with foetal tissue. Once these variables are understood and controlled, then patient selection and graft content and placement can be systematically tested to potentially generate highly consistent results within an appropriately selected patient population to provide very effective symptomatic relief. However, the disease will ultimately continue to progress and even this intervention will require a combined strategy to prevent further degeneration. Combinatorial strategies that use neuroprotection with local augmentation may be the key intermediary steps to a full cure through complete restoration of the affected neural systems.

(c) *Systems reconstruction*

The complete and perfect reversal of neurodegenerative disease requires that degeneration is halted and that authentic circuitry is reconstructed in multiple

systems. Systems reconstruction is arguably the most improbable use of stem cell transplants in early clinical strategies. Creating precisely patterned neurons of the correct subtype that survive and send projections to the appropriate target fields following transplant seems virtually impossible. With few exceptions, transplanted cells cannot do this in a normal brain, much less within the hostile context of the degenerating brain.

Although only recently validated as a natural biological process, neurogenesis in the adult brain is easily detected in two regions: the hippocampus and the olfactory bulbs. In both areas, intrinsic stem cell populations continuously generate neurons and glia. The neuroblasts migrate, sometimes very long distances, to their final destination where they differentiate and functionally integrate into pre-existing networks (Alvarez-Buylla & Garcia-Verdugo 2002; Gage 2002). Neurons destined for the olfactory bulbs migrate out of the subventricular zone of the lateral ventricle to the olfactory bulbs where they differentiate into granule layer and peri-glomerular interneurons (Lledo *et al.* 2004). Within the hippocampus, newborn neurons are produced adjacent to the granule cell layer and ultimately differentiate and integrate into this densely packed population of neurons (Kempermann & Gage 2000). In neurogenic areas, developmental processes are recapitulated and neural stem cells are able to generate progeny that contribute to the function of existing circuits.

Brain areas outside the hippocampus and olfactory bulbs are considered 'non-neurogenic' because reported neurogenesis is limited at best and controversial (Gould & Gross 2002; Spalding *et al.* 2005). However, stem cells residing in non-neurogenic regions do appear to produce neuronal and glial progenitors in response to injury such as stroke or ischaemia (Arvidsson *et al.* 2002; Parent *et al.* 2002). These progenitors migrate towards injury to initially repopulate the injured brain area. Unfortunately, new neurons are not produced in enough abundance at the site of injury to replace the number of mature neurons lost, and virtually all die within the weeks following injury.

Evidence does suggest that injury itself may modulate the ability of neural progenitor cells to differentiate and new neurons to survive as integrated neurons. In an elegant model of selective neuronal death, Magavi *et al.* (2000) show that neuronal death in the absence of gross anatomical damage is followed by direct neuron replacement in the adult cortex (Chen *et al.* 2004). These newly generated neurons send projections considerable distances to appropriately innervate target sites, suggesting that relatively intact tissue architecture is more permissive than previously suspected.

The concept that local permissive micro-environments are required for neuron production and integration has gained considerable support in the past five years (Alvarez-Buylla & Lim 2004; Ma *et al.* 2005). Endogenous stem and progenitor cells, isolated from non-neurogenic and neurogenic adult brain areas, can be re-implanted into multiple areas to determine how the brain itself instructs neural stem cell fate (Gage *et al.* 1995; Palmer *et al.* 1995). When transplanted into neurogenic areas, the stem cells generate neurons and glia. When transplanted into other areas, the stem cells generate only glia. The source of neurogenesis-promoting signals within these micro-environments is complex and includes intracellular signalling within the niche itself as well as signalling through both diffusible and circulating growth factors and hormones.

Within the hippocampal formation, the neurogenic niche is associated anatomically with the microvasculature (Palmer *et al.* 2000) and several studies implicate angiogenically stimulated endothelial cells as important sources of instructive signalling (Leventhal *et al.* 1999; Shen *et al.* 2004). Astrocytes and neurons strongly promote neurogenesis (Song *et al.* 2002; Deisseroth *et al.* 2004), and circulating growth factors and steroid hormones act either on the vascular cells of the niche or directly on the progenitors within the perivascular space to mediate activity-induced increases in hippocampal neurogenesis (Tanapat *et al.* 1999; Aberg *et al.* 2000; Fabel *et al.* 2003) or chronic stress-induced decreases in neurogenesis, respectively (Tanapat *et al.* 1998).

Hope that non-neurogenic areas could become injury-induced permissive environments for neurogenesis exists. Hippocampal CA1 region pyramidal neurons are exquisitely sensitive to ischaemia, and the entire lamina can be ablated by brief oxygen deprivation in rodents. Neural stem cells do not replace neurons in this animal model unless the mitogens fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF) are infused into the ventricles adjacent to the injured area; mitogen infusion dramatically activates stem cell pools to initiate a startling level of CA1 region repair (Nakatomi *et al.* 2002). Similarly, the abortive neurogenesis seen following stroke (Arvidsson *et al.* 2002; Parent *et al.* 2002) can be enhanced by erythropoietin application (Wang *et al.* 2004). While the mechanisms mediating these effects are unclear, the empirical outcome is promising for potential clinical stem cell strategies.

Why the adult mammalian brain does not invoke its own repair potential is unclear, but growing evidence suggests that the immunological response to injury creates environments that promote remyelination and glial scar formation over neurogenesis. In fact, the hippocampal dentate gyrus is extremely sensitive to inflammatory signalling and rapidly downregulates neurogenesis in response to cytokines associated with either systemic or local immune activation. With injury, the same signals that rapidly recruit glia to the injury site and remyelinate axons appear to potently divert stem cells from generating neurons. Interestingly, oral administration of non-steroidal anti-inflammatory drug (NSAID) can attenuate some of this signalling to restore hippocampal neurogenesis (Monje *et al.* 2002; Ekdahl *et al.* 2003; Monje *et al.* 2003). NSAID administration also appears to improve the survival of neuronal progenitors that migrate to non-neurogenic brain areas following focal ischaemic stroke (Hoehn *et al.* 2005).

Injury could induce neurogenesis by simply activating the local vasculature and, therefore, angiogenesis to produce an angiogenic niche (Imitola *et al.* 2004) that recruits migrating neuroblasts to the injury site, inducing neurogenesis (Arvidsson *et al.* 2002; Parent *et al.* 2002). However, the niche setup by injury-induced angiogenesis is influenced by pro-inflammatory signalling that makes the resultant neurogenesis abortive.

This pro-inflammatory signalling would need to be attenuated to permit neuroblast production and neuron survival (Hoehn *et al.* 2005), and signalling that promotes maturation and integration of correct neuronal subtypes would need to be amplified. The latter step remains an inscrutable black box that appears to require the seemingly impossible task of recreating exquisite developmental patterning within the hostile injury environment. However, some non-neurogenic areas are capable of reconstruction (e.g. pyramidal projection neurons of the hippocampus or cortex; Magavi *et al.* 2000; Nakatomi *et al.* 2002), suggesting that stem cell circuit reconstruction strategies may be possible. The steps towards testing this concept in endogenous or transplanted stem cell-mediated repair are fairly well defined.

5. MISSING LINKS AND NEXT STEPS

(a) *Cell programming strategies*

During development, sophisticated spatial and temporal cues pattern the final CNS networks. For example, dopamine neuron development has been well characterized. At mouse embryonic day 9.5, midbrain neuroepithelial progenitor cells express engrailed genes (En1 and En2) and homeobox transcription factors Lmx1b, Otx2 and GBX2. These midbrain progenitors commit to the dopaminergic lineage (upregulating TH) and enter a post-mitotic stage (E10–10.5) when exposed to sonic hedgehog (SHH) and FGF-8, concurrently migrating to populate the SNc and initiate target innervation. The transcription factor Nurr1 and homeodomain transcription factors Lmx1b, Pitx3 and En1/En2 are involved in these developmental processes (Lin & Rosenthal 2003; Simon *et al.* 2003; Wallen & Perlmann 2003).

Empirical evidence suggests that exposing stem cells to a few key patterning stimuli in a stepwise fashion can trigger neuron subtype-specific programmes. *In vitro*, a developmental dopamine neuron-like pattern of gene expression can be recapitulated to induce the generation of midbrain dopamine neurons from hESCs. Initially, undifferentiated ES cells are 'neuralized', either through aggregation which promotes spontaneous differentiation in embryoid bodies or by treatment with retinoic acid in co-culture with mesenchymal lineage cells, which provide potent neuralizing signals that favour dopamine neuron production (for review see Sonntag *et al.* 2005). The neuralized cells differentiate into dopaminergic neurons following exposure to FGF-8 and SHH, which are at least similar to midbrain DA neurons in that they can integrate into CNS tissue and provide limited functional benefit in a rodent PD model (Ben-Hur *et al.* 2004). hESCs can also generate lower motor neurons that could attenuate the motor deficits observed in the animal models of ALS (Singh *et al.* 2005).

Although DA or motor neurons generated *in vitro* closely resemble their *in vivo* counterparts, several caveats remain. Patterning in a dish is not as complete as patterning in tissues and upon transplantation, hESC-derived cells neither survive nor maintain their phenotype as well as authentic neurons obtained from foetal tissue (Bjorklund *et al.* 2002; Ben-Hur *et al.* 2004). In addition, *in vitro* patterning does not prevent the generation of unintended neuronal subtypes; a significant portion of cells that survive transplant do not express the desired markers (Mendez *et al.* 2005).

hESC-derived transplant heterogeneity may be either beneficial or detrimental. Foetal tissue grafts do contain heterogeneous cell types, such as neurons, glia and vascular cells. These accessory cells could provide important patterning cues for the desired mature neuronal phenotype and probably also provide trophic support. Le Belle *et al.* (2004) have demonstrated that boosting energy stores among glia in these heterogeneous grafts *ex vivo* supports neuronal survival following transplant.

Once a transplant population of ideally patterned progenitors is generated, axon guidance and connectivity remain significant conceptual barriers. Decades of work in spinal cord injury paints a dreary picture for generating long-distance connections within an injured environment (Kraus 1996; Schwab & Bartholdi 1996). However, there is progress in identifying barriers, such as myelin-associated inhibitory cues (Fouad *et al.* 2001; Grados-Munro & Fournier 2003; McGee & Strittmatter 2003) and developing strategies to circumvent them (for reviews see Bunge & Pearse 2003; Verma & Fawcett 2005). One relatively unexplored research avenue would identify whether adequate guidance cues for correctly guiding appropriate connectivity of correctly patterned neuroblasts persists in the adult brain. We do know that when neurogenesis occurs spontaneously in the adult in the hippocampus and olfactory bulb, or is induced by the selective ablation of hippocampal or cortical projection neurons, correct long-distance projections are generated (Gould & Tanapat 1997; Magavi *et al.* 2000; Nakatomi *et al.* 2002; van Praag *et al.* 2002). Whether this phenomenon is generalizable to an injured environment that includes traumatic injury of gross necrotic degeneration is unclear. Regardless, the study of axon guidance and connectivity cues in both the intact and injured adult CNS seems to be the next critical frontier in neurological repair.

Finally, critical safety issues relating to the efficiency of differentiation and patterning require resolution before stem cell strategies move to clinic. Heterogeneity in the cultured stem cells' response to patterning cues can be detrimental when some cells fail to differentiate and retain the aggressive growth characteristics of the pluripotent hESC. hESCs readily form teratomas upon implantation in animals. This somewhat disturbing attribute is natural for stem cells and teratoma formation is a common assay used to determine lineage potency of a hESC line (can the hESCs generate ectoderm, mesoderm and endoderm derivatives?). Early hESC transplant work demonstrated hyperplasia or frank tumour formation, relatively frequently (Bjorklund *et al.* 2002). More recent strategies demonstrate that fully differentiating hESC populations prior to transplant or sorting to either deplete undifferentiated cells or to selectively ablate those cells which might escape differentiation signals and continue growing (Arase *et al.* 1999; Singh *et al.* 2005) appear to improve the transplant safety in animal models.

(b) *Immunology of cell replacement*

For more than 50 years, the CNS has been considered 'immunologically privileged' because graft survival is prolonged relative to graft survival in extra-CNS sites. The CNS was thought to exhibit this privilege because blood–brain barrier protection, antigen-presenting cell paucity and limited lymphatic drainage prevented efficient adaptive immune response activation. Because cellular grafts survive long term in the human CNS without immunosuppressant therapy (Freed *et al.* 2001), few transplant studies have fully assessed the role of inflammation in graft survival or long-term function (for excellent reviews on mechanisms governing graft rejection see Barker & Widner (2004) and Bradley *et al.* (2002)). However, post-mortem examinations of foetal mesencephalon-derived graft recipients (independent of the post-transplant immunosuppressant regime) reveal activated microglia and host immune cell infiltration at the host–graft interface and throughout the graft (Freed *et al.* 2001; Olanow *et al.* 2003), strongly suggesting that inflammation compromised graft survival and functional outcome. The lack of attention to the role that inflammation will play in transplanted stem and progenitor cell behaviour is exemplified by the view that they exhibit little immunogenicity, but studies are beginning to show that this view is outdated; mature differentiated derivatives of stem cells that are needed for therapy have normal histocompatibility antigen expression profiles (Drukker *et al.* 2002).

The mechanisms that will induce human stem cell-derived graft rejection are the same that induce the rejection of organs and other transplanted tissues. ABO blood-group antigens are expressed by virtually every cell in the body. Hosts who do not express A and B antigens develop cross-reactive antibodies specific for the non-expressed antigens that bind to donor tissue to produce hyperacute graft rejection. The classical MHC antigens consist of human leucocyte antigens (HLAs), HLA-A, HLA-B and HLA-C (MHC-I class molecules) that are expressed on all nucleated cells in the body and HLA-DR, HLA-DQ and HLA-DP (MHC-II class molecules) that are expressed on antigen-presenting cells such as dendritic cells, B cells, macrophages and thymic epithelial cells as well as reactive microglia and astrocytes in the injured brain. The probability of allograft rejection increases with the degree of HLA mismatch between host and donor. A zero mismatch grade (all six MHC antigens are matched) yields good graft survival. A mismatch grade of six (complete mismatch at both alleles of HLA-DR, HLA-A and HLA-B) requires lifelong immunosuppressive therapy to prevent rejection.

Cells transplanted into parenchyma survive better than those transplanted periventricularly (Barker & Widner 2004), potentially owing to proximity to infiltrating antigen-presenting cells near periventricular zones. The probability of immune system-mediated graft rejection also increases with the degree of trauma produced by the transplant procedure. Nikkhah *et al.* (1994) demonstrated that up to 20-fold more foetal dopamine neuroblasts survive when transplanted in submicrolitre suspensions over multiple implantation sites using a glass capillary 10-fold smaller than a Hamilton syringe at the tip of the syringe-driver instrument. This procedure significantly reduced the signs of inflammation also. Foetal cells transplanted as solid chunks are more immunogenic than when transplanted as suspension grafts, probably because donor blood capillaries contained in chunks express high MHC-I levels (Winkler *et al.* 2005).

Therefore, HLA matching, meticulous technique and immunosuppressant therapy will probably be required to ensure good survival of grafted human stem cells or their more differentiated progeny. For example, Krabbe's and Hurler's syndrome patients transplanted with partially HLA-matched grafts (four to six of six HLA loci) exhibit excellent survival and good CNS engraftment. For each recipient, multiple donors were screened to ensure partial HLA compatibility, absence of infection and high enzyme activity (Staba *et al.* 2004; Escolar *et al.* 2005). This level of screening may be sufficient to prevent the rejection of relatively primitive human stem cell grafts in biopump strategies, but more stringency may be required for strategies that require the transplant of differentiated stem cell progeny. Drukker *et al.* (2002) have demonstrated that MHC-I protein expression in hESC derivatives increases dramatically upon differentiation or upon exposure to the pro-inflammatory cytokine interferon-γ.

Neuroinflammation is now considered a hallmark of neurodegenerative disease, and the survival of transplanted human stem cells or their pre-differentiated progeny will probably be compromised by donor/host HLA mismatch. Perfect HLA compatibility is desirable but extremely difficult to achieve using an unrelated donor strategy. For example, perfectly HLA-matched donors can only be located for approximately 75% of bone marrow recipients and there are more than seven million HLA-typed volunteer bone marrow donors registered worldwide (Bradley *et al.* 2002).

Somatic cell nuclear transfer (SCNT), or therapeutic cloning, would circumvent host immune system rejection by perfectly HLA-matching donor cells for transplantation. Hwang *et al.* (2004) report that they could therapeutically clone cells, which generated great excitement among the stem cell research community. They claimed that they could inject a single fibroblast taken from a skin biopsy into an enucleated human egg and then mimic fertilization by electrically stimulating the egg. The engineered cell generated approximately 200 cells within a few days, some of which exhibited the pluripotent characteristics of hESCs. Because these pluripotent cells contained the nuclear DNA of the skin cell donor, they would be completely immune-compatible cells that could be transplanted back into the skin cell donor. This report has since been retracted, but the prospect of generating personalized cell lines for transplantation is exciting, given that most patient deaths are immune response-related. Hopefully, the technique will be legitimately refined in the near future. The procedure does require the donation of eggs, considered morally unethical in many societies, but ongoing work to explore synthetic nuclear reprogramming strategies will ultimately generate more widely accepted technologies.

Studies investigating the safety and efficacy of transplanted neural tissue have generated little

consensus about the need for immunosuppression, and there is a dire paucity of information about immunologic interactions between host and stem cell-derived transplants (Bradley *et al.* 2002; Barker & Widner 2004). Experience with foetal tissue in clinical trials suggests that outcome is improved with immunosuppressant therapy. Although the brain is compromised in its ability to mount full-fledged graft rejection, inflammation and immune–brain interactions run well beyond simple cell survival. Reactive gliosis and microglial activation are hallmarks of virtually all neural insults including neurodegenerative disease (Block & Hong 2005; Marchetti & Abbracchio 2005), and ongoing inflammatory status is likely to influence both the survival and the ultimate fate of transplanted hESC derivatives.

The second and, perhaps, more important immunological interaction concerns the activation of the innate immune response which can significantly impact the success or failure of a transplant even before graft rejection mechanisms are activated. Foreign antigens are irrelevant in innate response which is potently activated by something as simple as injection cannula damage as well as by ischaemia or disease-related cell death.

The innate CNS immune response is predominantly maintained by local activated microglia, but astrocytes and neurons can also mediate immune signalling following injury (Land 2005). These reactive cells produce an array of pro-inflammatory cytokines and chemokines that, in turn, recruit circulating leucocytes to the injury site. Invading macrophages and leucocytes then mediate the adaptive response in which foreign- or self-antigen recognition triggers T and B cell activation and amplification that induces cellular or humoural cytolysis. When histocompatibility antigens are poorly matched, this adaptive response can lead to graft rejection. However, inefficient amplification of cytolytic T cells in the brain does permit the long-term survival of mismatched grafts in the absence of immunosuppressant therapy (Freed *et al.* 2001). However, the long-term clinical consequence of this scenario is undetermined.

Although graft survival is the paramount concern in transplantation, published evidence demonstrates that surviving grafts will be influenced by the innate immune response. In rodents, the innate immune response significantly impacts the survival and fate of both endogenous and transplanted neural progenitor cells. In the adult hippocampus, inflammation potently inhibits the ongoing production of neurons and reduces the survival of those few neurons that are produced (Monje *et al.* 2002, 2003; Ekdahl *et al.* 2003), even swaying the fate of neural progenitor cell grafts towards astrocyte and oligodendrocyte lineages. In terms of a transplant strategy, this effect would be detrimental for reconstructing neuronal circuitry. On the other hand, activation of the innate response may be beneficial for replacing glia.

Activation of the innate immune response activation may also be beneficial, stimulating the migration of transplanted human neural progenitor cells towards the sites of inflammation. For example, multipotent neural progenitors express the chemokine receptor CXCR-4 and transplanted cells have been shown to migrate to sites where astrocytes and endothelia upregulate the CXCR-4 ligand, stromal cell-derived factor 1α (Imitola *et al.* 2004). This property may be particularly advantageous for distributing human stem cell-derived biopumps or pre-differentiated cells throughout a degenerating area.

How the immune system will interact with outcome of a transplant strategy requires careful consideration. Immune response modulation may be extremely important for PD, HD, AD and ALS strategies where neuronal replacement is desired, less important for MS strategies where oligodendrocyte production is required and even undesirable in strategies where cell biopump dispersal is required.

(c) *Accurate demonstration of efficacy in animals*

Predicting how transplant-mediated functional improvements in animal neural networks will translate to human neural networks is difficult. Although rodent models of many human degenerative diseases exist, the acute mode of toxin-induced cell death or the gross over-expression of a human gene does not perfectly mimic the chronic nature of the human disease. Then, there is the problem of scale. The human brain is much bigger than the mouse brain with relatively longer projection distances and more complicated neural network dynamics. In fact, fine cognitive function is impossible to measure in animals.

Effective preclinical investigation of stem cell therapy safety and efficacy will require the development (or refinement) of animal models that more accurately reproduce the human condition. Animal models of each of the principal degenerative diseases come with some limitation. Experimental PD work exemplifies the complexity of testing the safety and efficacy of strategies prior to clinical use. In an excellent review, Beal (2001) proposed that an optimal animal model of PD should exhibit five characteristics, and we have generalized these to encompass all major neurodegenerative disease models.

First, the animal is born with the appropriate number of disease-vulnerable cells (SNc A9-type dopaminergic cells in PD models), which selectively, gradually and quantifiably degenerate. Second, as degeneration progresses, the animal should exhibit readily measurable functional impairment that includes the cardinal symptoms of the modelled disease (bradykinesia, rigidity and resting tremor in PD models). These attributes should be consistent across a variety of behavioural tasks and correlate with the extent of denervation. Third, the model should show the complete characteristic pathology associated with the disease (neuron death, Lewy body inclusions and inflammation in PD models). Fourth, models of human single-allele genetic disease should be based upon a single genomic mutation to allow for robust propagation and crossing with enhancer/repressor strains. Finally, and perhaps arguably, Beal (2001) promotes the concept that functional impairment should progress quickly to permit the rapid and economic screening of treatment efficacy. This is obviously problematic given the potential differences in the immune–brain interaction that might exist in

very slow progressing human diseases versus rapid cell death in the 'ideal' animal model; yet, admittedly rapid screening is critical for driving advances. We would add that an ideal model is robust in both rodents and primates to facilitate the scaling of effective therapies from preclinical to clinical contexts.

Current animal models of human neurodegenerative disease have been invaluable for surgical and pharmacological screening, but shortcomings in their replication of key disease characteristics may underlie the failure of several promising transplant clinical trials. Our understanding of PD, for example, has been facilitated by the discovery of toxin-induced parkinsonism and gene mutations underlying rare familial cases of PD. The three major toxin models of PD are produced by unilateral 6-hydroxydopamine (6-OHDA) injections, intraperitoneal 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injections or intravenous rotenone (mitochondrial Complex I1 inhibitor) injection.

The 6-OHDA model of PD is advantageous because its retrograde transport selectively kills nigral dopamine neurons in rats, mice, cats and primates, which can be assayed by apomorphine- and amphetamine-induced rotatory behaviour (Ungerstedt 1971) and produces easily measured functional deficits (Olsson *et al.* 1995). However, 6-OHDA-induced degeneration is acute (more so when injected into the medial forebrain bundle versus striatum; Sauer & Oertel 1994) and Lewy bodies and ubiquitinylated intracytoplasmic inclusions are absent (Beal 2001).

The rotenone model of PD is advantageous because nigrostriatal dopamine neurons degenerate progressively coincident with a loss of TH, dopamine transporter and vesicular monoamine transporter immunoreactivity and the development of Lewy body-like inclusions. Rotenone-treated rats exhibit many of the cardinal PD symptoms, which improve after apomorphine treatment. However, rotenone induces PD-like symptoms in only approximately 50% of treated rats (Betarbet *et al.* 2000).

Parkinsonism in drug addicts is caused by synthetic heroin contaminated with MPTP, which is catalysed to 1-methyl-4-phenylpyridinium (MPP^+), a potent Complex I inhibitor (Langston & Ballard 1983). MPTP induces nigrostriatal dopamine cell death in rodents and primates, and in primates best replicates the clinical signs of PD (Burns *et al.* 1983). The main disadvantages of the MPTP model are that degeneration is relatively acute and, normally, Lewy body inclusions are only observed in aged primates (Beal 2001). Toxin models have been ungainly for assessing the efficacy of biopump-delivered neurotransmitter-synthesizing enzymes or neuroprotective agents, because the speed and the degree of cellular destruction leaves little intact circuitry (Costantini *et al.* 2000).

Many genetic mutations are now evident in familial cases of PD (Lewthwaite & Nicholl 2005). Genetic PD models include α-synuclein knockout mice and transgenic mice over-expressing the mutant human α-synuclein protein. The knockout mice are viable and develop normally, but exhibit a weak PD phenotype (Abeliovich *et al.* 2000). Transgenic α-synuclein over-expressers exhibit dopamine system degeneration and locomotor impairments of varying degrees depending upon the promoter used to drive expression; however, selective loss of dopamine neurons is not observed (Orth & Tabrizi 2003).

The lack of an exact neurodegenerative disease phenocopy in animal populations continues to pose problems for assessing the efficacy of early versus late intervention with stem cell (and other) therapies. One clear example was the emergence of transplant-induced dyskinesias in a significant number of PD patients transplanted with foetal mesencephalon-derived neuroblasts. This was not predicted from rodent models and clearly demonstrates the pitfalls associated with pioneering efforts in cellular therapy.

Despite their shortcomings, animal models do replicate some key features of human neurodegenerative disease. However, whether human and animal ESCs are fully identical and whether hESC derivatives will perform equally well in animals and humans is currently unclear. Xenograft immune responses versus allograft responses are distinct and may complicate the interpretation of preclinical toxicity and efficacy studies using clinical grade hESCs.

6. DISCUSSION

We argue that a multifaceted approach to treating human neurodegenerative disease could drastically improve symptomology and overall quality of life, and that human stem cell-derived grafts generate an excellent foundation for this approach. The first and, perhaps, simplest approach would be to use stem cell-derived biopumps to deliver neuroprotective agents locally to slow or stop the degenerative process. This approach has already shown excellent promise in clinical trials for human disease. Second, cell biopumps could augment degenerated CNS circuitry output, either by delivering neurotransmitter in bulk via non-neuronal cells or by the local synaptic modulation provided by ectopic transplants of ESC-derived neurons of the appropriate subtype. Finally, the degenerated neurons could be entirely replaced by human stem cell progeny that have been directed to differentiate down a desired cell type lineage. Regenerating long-distance projection neurons may take time and require one or more adjunct therapies to modify local guidance cues. In all cases, the post-transplant survival of phenotypically appropriate cells will need to be improved, perhaps by ensuring that cells for transplant are HLA matched and by modulating the host immune response with immunosuppressant therapy.

Effective but safe cell doses will need to be established in primate models optimized to more accurately mimic the human condition. For example, neurotrophic factors protect cells from death at optimal doses but induce cell death at high doses (Friedman 2000). Side effects must also be monitored carefully. The generation of non-human primate ESC lines is an exciting prospect for preclinical modelling of stem cell therapies that will permit evaluation of the safety, feasibility and efficacy of combined stem cell therapies in either allograft contexts or isograft contexts using nuclear transfer-derived ESC lines.

(a) *Stem cell therapy imminence*

Neurodegenerative disorder pathologies vary in the complexity and focality of cell death and therefore in their array of symptomology. The imminence with which a stem cell strategy reaches clinic will probably correlate negatively with this complexity and positively with how optimally animal models mimic the human disease. PD and HD are perhaps the most thoroughly modelled neurodegenerative diseases and are associated with relatively focal early lesions and relatively specific affected neuronal subtypes. Neuroprotection and augmentation strategies have already shown very promising preclinical and clinical results, at least in a few patients. Full regenerative strategies will be more difficult for PD owing to the long distances that SNc axons travel to the striatum and scant knowledge about how this process is controlled and/or modified in the adult, diseased brain.

Stem cell therapies for ALS are also fairly imminent and a group lead by Svendsen (Klein *et al.* 2005) at the University of Wisconsin is driving forward with a clinical trial to test the safety and feasibility of transplanting GDNF-secreting foetal neural precursors into the spinal cord of ALS patients. Stem cell therapies for MS and AD will require more preclinical development based upon the diffuseness of their aetiology and the lack of strong animal models.

(b) *Scientific, ethical and political considerations*

hESC strategies appear to hold higher potential for treating human neurodegenerative disease over foetal- or adult-derived cell strategies because hESCs can be quickly expanded and generate a wider variety of cell types. However, their use in research and medicine has been as hotly debated as the use of aborted foetal tissue. While Britain has developed rational guidelines that allow research to progress under close ethical and moral scrutiny, the US has not generated guidelines for hESC research and governmental funding has been limited to studies involving 22 federally approved hESC lines that were created before August 2001 (see: http://www.stemcells.nih.gov/research/registry). The ethical and political issues have even plagued the US private sector as venture capital has been reluctant to invest in technologies that may at the whim of the conservative minority become increasingly difficult to work with (Giebel 2005). This uncertainty is potentiated by intellectual property rights held by the Wisconsin Alumni Research Foundation (Rabin 2005) which claims ownership of all ES cells and their derivatives.

Similar political and religious disagreements plague stem cell research worldwide. In Europe, four different models are emerging. First, the UK model permits the generation and use of hESCs as well as therapeutic cloning, with certain restrictions. Second, the Netherlands model permits the generation and use of hESCs but forbids therapeutic cloning. Third, the German model forbids the generation of hESCs and therapeutic cloning but allows, under exceptional conditions, the use of existing lines for research. Fourth, in Ireland and Austria, the generation and use of hESCs and therapeutic cloning is forbidden. hESC research in these countries and the US lags behind the UK, Singapore, Israel, South Korea, China and Japan, which receive full government support for conducting hESC research (Bongso & Richards 2004).

Developing effective stem cell-based therapies is the only way to end this debate. While we have discussed cellular transplantation as one avenue of stem cell research, the opportunity to generate SCNT stem cell lines from patients who carry simple or complex genetic traits will provide an untapped and immense opportunity for discovery research in the form of disease-specific screening tools for drug development. For example, an array of 100 or even 1000 SCNT lines derived from PD patients could be used to generate abundant midbrain neurons. Millions of miniaturized cellular arrays could be used to screen for toxins that selectively kill diseased cells and, more importantly, to screen for drugs that selectively protect these cells from their unique toxin sensitivities. Identifying gene combinations that yield a disease predisposition would provide wonderful new insights into aetiology and eventually common causes and patient-specific interventions.

(c) *Ready for prime time?*

Embryonic stem cell sciences are a young and vigorous discipline discovered only in 1998 (Thomson *et al.* 1998). Considering the abundant clinical applications generated by the past 25 years of haematopoietic stem cell study, one would argue that widespread effective clinical applications generated by hESC technology will become mainstream over the next decade. Neurological repair targets range from simple enzyme replacement in neonatal storage diseases to more complex systems restoration following extensive trauma and necrotic tissue loss. The first application to reach clinic is unclear, but current strategies for simple enzyme replacement, remyelination and localized neuronal augmentation are approaching clinic-level safety and efficacy goals in animal models. Clearly, safety is of primary concern when using cells that intrinsically form tumours, but recent rodent studies show no evidence of adverse effects (Keirstead *et al.* 2005; Tabar *et al.* 2005). In the US, federally required preclinical toxicity and safety studies are being initiated for spinal repair stem cell strategies and rumours of advances in Pacific Rim countries suggest that clinical stem cell applications may come to light even as this article is being prepared for publication.

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