Future Medicine

Regenerative Medicine

Neonatal desensitisation for the study of regenerative medicine

Summary

Cell replacement is a therapeutic option for numerous diseases of the central nervous system (CNS). Current research has identified a number of potential human donor cell types, for which pre-clinical testing through xenotransplantation in animal models is imperative. Immune modulation is necessary to promote donor cell survival for sufficient time to assess safety and efficacy. Neonatal desensitisation can promote survival of human donor cells in adult rat hosts with little impact on the health of the host and for substantially longer than conventional methods, and has subsequently been applied in a range of studies with variable outcomes. Reviewing these findings may provide insight into the method and its potential for use in pre-clinical studies in regenerative medicine.

Keywords: Transplantation, rodent models, cell replacement, Huntington's disease, immunosuppression, xenograft, desensitisation, rejection

Why is long term assessment of human neural grafts in rodent hosts needed?

Regenerative medicine seeks to provide therapies for a wide range of diseases that are associated with the loss or dysfunction of a specific population of cells. Two candidate neurological conditions with relatively focussed loss of specific neuronal populations are Parkinson's disease (PD), in which there is loss of dopaminergic projection neurons in the substantial nigra, and Huntington's disease (HD) in which there is loss of medium spiny projection neurons (MSNs) from the striatum. Implantation of primary foetal cells (PFCs) taken directly from the developing brain has provided proof of concept that transplantation has the potential to be an effective therapeutic option in PD and HD in both animal [1-3] and clinical studies [4, 5]. However, PFCs are not a practical option for routine use in the clinic for several reasons. In particular, in order to implant sufficient cells of a specific striatal or dopaminergic phenotype several foetal donors are necessary for each patient (potentially up to 6 in HD, and 8-12 in PD) [6, 7]. As storage of PFCs prior to transplantation is currently limited to a maximum of 8 days in hibernation medium [8], clinical transplantation relies on the availability of sufficient donor tissue within this time window. Furthermore, as patients receive transplants from different foetal donors, issues can arise with the variability and purity of the cells that may affect the success of the transplants [7, 9, 10]. Additionally, the risk of a graft containing cells that are immunologically incompatible is raised (see below).

Thus, as well as refining the transplantation methodology, current research aims to identify sources of potential donor cells that will offer a practical alternative to PFCs; which is most likely to be one or more of the stem cell sources [11]. It will be important that such cells can produce large stable populations of clinical grade cells that can be quality controlled and standardised across transplants. However, an equally important requirement of donor cells is that they are able to recapitulate the precise features of the cells that have been lost to the disease process [12]. For example, for PD this requires the transplantation of cells which can not only generate a dopaminergic phenotype, but will specifically become dopamine cells of the A9 midbrain phenotype. For HD, this means generating

cells of a MSN phenotype that possess all the features of the native cells which arise from the foetal whole ganglionic eminence (WGE) and are able to make appropriate synaptic connections post transplantation. Not surprisingly, developing effective protocols that achieve this and can be translated for GMP (good manufacturing practice) cell production is difficult and usually requires months/years of protocol refinements *in vitro* for a single cellular phenotype. Although it is possible to use cellular markers *in vitro* to assess several aspects of the differentiated cellular phenotype, ultimately it is necessary to transplant cells into animal models of disease to allow the cells to develop fully, make connections with appropriate neuronal inputs and outputs, and display their full mature phenotype. Indeed, although there are many reports of stem cells being manipulated to express specific neuronal phenotypes *in vitro*, very few have shown convincing comprehensive behavioural efficacy in animal models. Furthermore, assessments must be made to ensure that the donor cell populations do not contain small numbers of cells which may be capable of tumour formation.

There are practical issues associated with transplanting human cells into the rodent brain. Although the brain is recognised as a relatively immune privileged site, it is clear that this is not absolute [13] and human cells in the rat brain will be rejected over a period of approximately three weeks without effective modulation of the host immune response [14].

Commonly used methods of immunosuppression

Immunosuppressant drugs such as Cyclosporine A (CsA), Tacrolimus (FK-506) and Sirolimus (Rapamycin) are commonly used to promote survival of xenografts in rodent hosts. Using one or a combination of these drugs or additional compounds can promote survival of human cells transplanted into the CNS and may be used to prevent xenograft rejection. Immunosuppression with daily injections of CsA is frequently used in rodent hosts. We have found up to 75-80% survival of neural xenografts in rat hosts immunosuppressed with CsA for up to a maximum of 20 weeks post-transplantation [15]. Variability in xenograft survival with the same treatment has been reported in some studies [16, 17], suggesting inconsistencies amongst different host animals and following the transplantation of different cell types. In particular, although reliably promoting the survival of human xenografts in the rat brain, we have found the same treatment to be much less reliable in promoting survival of human transplants in the mouse brain [18] and it is possible that the use of an increased dose of CsA may be required in this species [17]. CsA treatment demands daily injections that are stressful to the animal and, with long term use, result in severe adverse side effects including renal toxicity [19, 20]. Although immunosuppressive treatment is useful for relatively short term evaluation of graft survival and functional improvement, beyond 16 weeks animal health begins to deteriorate, precluding functional testing up to full differentiation of transplanted human cells, which can take approximately four times longer than rodent cells to reach maturity [21, 22]. Thus realising the true potential of the cells is prevented in this model.

There have been a number of reports of successful tolerance to transplants following blocking of costimulatory molecules. In a recent study in adult murine hosts, three costimulatory receptor blocking antibodies (CTLA4-Ig, Anti-LFA-1 and anti-CD40L) were administered at 0, 2, 4 and 6 days post-transplantation to induce tolerance to xenogeneic human ESC and iPSC derived transplants [23]. This treatment successfully prevented rejection compared to no treatment or tacrolimus and sirolimus treatment. Mouse hosts were tolerant to donor cells, exhibiting T cell anergy and no

detrimental effects on the hosts' immunity to other cell types were observed [23]. Although offering a potential method of avoiding immune rejection; survival so far has only been demonstrated up to 8 weeks post transplantation [23].

Immune compromised rats or mice are common transplant hosts, since they lack the ability to mount an immune response to xenografted tissues. A number of useful host models exist, in both rats and mice, for the assessment of donor cell differentiation and integration. The long term assessment of grafted cells *in vivo* is necessary to investigate any changes which may not be detected in short term survival studies afforded by immunosuppressant drug treatments. To this end, the phenotype of transplanted human PFC- and ESC- derived cells has been investigated *in vivo* by several groups using athymic nude rats [24-26]. For example, Aubry and colleagues transplanted human ESC-derived striatal progenitors into immunocompetent hosts treated with conventional immunosuppression to assess short term graft survival, and into immunodeficient hosts to study long term cell maturation and integration [27]. Positive results regarding their differentiation protocol were identified in the short term, however longer term assessment in nude rats revealed overgrowth by around 2 months post-transplantation [27]. This would not have been identified with only short term assessment of graft development, thus highlighting the value of a model which permits adequate assessment of the maturation of grafts in the long term. Numerous immunodeficient mouse models also exist including; severe combined immunodeficient (SCID) mice and *Rag1* or *Rag2* knockout mice; which lack mature B and T lymphocytes respectively [28, 29] thus tolerating human transplants without rejection.

However, histological assessment of the graft is not by itself sufficient, and it is important that evaluation of grafts also includes functional assessment. Due to their susceptibility to infection and vulnerability with repeated handling, immune compromised hosts are problematic for behavioural testing.

Another strategy for achieving xenograft survival is to transplant into the early neonatal period while the immune system is still immature and before the circulation of mature T cells [30]. Neural xenotransplants, e.g. of mouse or human tissue are known to survive long term in the neonatal rat brain when delivered up to 8 days of age, although beyond this point transplants are rejected [31, 32] due to maturation of the host immune system. Although the survival of such grafts is unstable, with a peripheral immunological challenge of an i.p. injection of xenogeneic donor cells or damage to the blood brain barrier (BBB) resulting in rejection [33], this can provide a useful model for testing donor cells, allowing *in vivo* assessment of safety and differentiation of human donor cells in the rodent brain or retina [34-36]. As discussed later however, the privilege of neonatal transplant survival has not been replicable in mouse hosts [37]. The neonatal brain cannot be considered to be comparable to the adult brain, since it provides a more permissive environment with the presence of more developmental signals. Findings may therefore not be representative of the therapeutic situation, in which immature cells are delivered to the adult brain.

Although these methods can provide a number of potential approaches for allowing the survival of human xenografts in rodent disease models, none provide a solution in which full graft maturation and functional efficacy of transplanted cells may be assessed long term. Therefore an alternative method is required for adequate testing of potential human donor cell types for transplantation.

Neonatal desensitisation as a method for assessing xenografts in the CNS

Cells implanted to the early neonatal rat brain appear to be recognised as "self" and are not rejected even once the immune system has matured [31, 32]. Based on this phenomenon we explored whether injecting human cells peripherally in the early neonatal period would allow the survival of similar cells subsequently transplanted into the brain in adulthood. We demonstrated that an intraperitoneal (i.p.) injection of a suspension of human PFCs from the developing cortex hPF(ctx) into a neonatal rat promotes the survival of a subsequent striatal transplant of human PF brain without further immunosuppression [15]. A schematic of this method is outlined in Figure 1. Neonatal injections were found to provide optimum survival when delivered between postnatal day (P)0 and P5; those injected at P10 or as adults did not support the survival of subsequent neural transplants. Crucially, survival of human cells was demonstrated until at least 40 weeks post-transplantation following desensitisation and transplantation with a range of different tissue types. Graft survival rates in desensitised hosts were also found to be comparable to hosts treated daily with CsA (~62-87% and 75-77% respectively, see Table 1), although survival in CsA immunosuppressed hosts could only be assessed up to 12-16 weeks due to poor health of the animals [15].

The mechanisms by which neonatal desensitisation protects adult neural xenografts from rejection by the host immune system are currently unclear. A similar phenomenon has been previously described with the induction of neonatal allograft tolerance in mice [38, 39]. Allograft tolerance has been achieved in mice following the injection of spleen [40, 41], liver [42], or bone marrow cells [43] in the neonatal period, in the same way as we have demonstrated with neural xenografts [15]. It is thought that the induction of neonatal tolerance requires the persistent presence of donor antigen during the neonatal period to expose the developing immune system to donor cells during the determination and recognition of self-antigens, resulting in recognition of both donor and host tissues as self [44]. However since the cells injected into neonatal rats for neonatal desensitisation

148 are from a different foetal donor to those used for adult transplants, much more variability will exist
149 between the donor antigens to which the developing immune system is exposed and those which
150 are delivered to the adult host. No tolerance to a specific antigen can therefore be assumed, making
151 the mechanisms behind effective desensitisation to neural tissue transplants from a range of donors
152 unclear.

Neonatal desensitisation in the rat host

The desensitisation method is now routinely and reliably used for transplantation experiments in our lab to test the maturation and functional efficacy of human xenografts in rodent models of both PD and HD (summarised in Table 2). In the majority of cases host animals were injected neonatally (i.p.) with a suspension of $1x10^5$ hPF(ctx), since this is the most abundantly available hPF tissue type. Successful application has been demonstrated in both Sprague Dawley (SD) and Lister Hooded (LH) rat hosts using lesion models of both PD and HD. Survival of hPF(ctx), hPF(WGE), ventral mesencephalon (hVM), and human embryonic stem cell (hESC)-derived neurons have all been found following desensitisation with hPF(ctx), and survival is comparable to that of transplants in hosts immunosuppressed daily with CsA. However, human foetal cells are not widely available and thus it was important to assess whether human cells of a non-foetal origin were capable of inducing desensitisation. As can be seen from Table 2, desensitisation was successfully induced following neonatal injections of non-foetal cells such as FNP-derived and ES-derived neuronal progenitors, and even non-neural cells.

There is some variability in the percentage of grafts surviving in both desensitised and CsA treated hosts that is as yet unexplained. Some of this may be due to poor viability of the donor cells, but it may also be the case that a strong mismatch between the cells used for desensitisation and those subsequently transplanted in adulthood, so that the latter are not adequately protected. For example, Table 2 shows that in host animals that were desensitised with hPF(ctx) cells from embryo A and subsequently then grafted with cells from embryo C, the survival of grafts is poorer than when the grafts are from embryo D, suggesting that A and C may be less well matched than A and D. Currently the numbers of animals included in such comparisons is too low to draw firm conclusions, but this sort of analysis may provide important insight into the mechanisms behind neonatal desensitisation and deserves further investigation. An additional caveat of these studies which may have caused variability in the data from desensitisation experiments is the use of outbred rat stocks

(SD and LH). Differences in MHC haplotype between host animals may result in different host responses to transplanted human donor cells. These studies therefore require replication using an inbred rat strain to reduce this variability and allow firmer conclusions to be reached from the data. An inbred strain that is suitable for behavioural studies would be optimal, in order to provide hosts which can be successfully desensitised, transplanted and undergo a battery of motor and cognitive behavioural tasks.

A number of other groups have used desensitisation to promote survival of various human cell types in animal models of disease. For example, rat hosts with induced knee cartilage defects were successfully desensitised with $1x10^5$ hESC-derived mesenchymal stem cells (hESC-MSC) [45]. These hosts could support survival of a collagen bilayer scaffold seeded with hESC-MSC for at least 8 weeks post-transplantation, whereas animals that were not desensitised neonatally showed increased T cell infiltration and transplant rejection [45]. Another group aimed to determine whether human Müller glia stem cells have the potential to differentiate into retinal ganglion cells (RGC) when transplanted into a LH rat model of RGC depletion [46]. In order to test this, survival of transplanted human Müller glia stem cells was achieved using a combination of neonatal desensitisation with $1x10^5$ cells of the same type, and daily administration of oral CsA, prednisolone and azathioprine. Hosts were transplanted at 3-4 weeks of age with undifferentiated stem cells or RGC precursors, both of which were found to survive under these conditions up to 4 weeks post transplantation [46]. Subsequently this group has also successfully assessed the survival of photoreceptors derived from hMSC following transplantation into rats, with the use of the same desensitisation and immunosuppression method [47].

Although neonatal desensitisation has been successfully used to promoting survival of various human cell types in rat hosts, there have also been some negative reports [16, 48]. Human cord blood derived neural stem cells (HUCB-NSC) were used for desensitisation of Wistar rats at birth. Neural transplants of HUCB-NSC were delivered to 6 week old desensitised hosts and survival

assessed at various time-points post transplantation. The presence of human cells was confirmed at early time-points (1 and 3 days after transplantation), and a substantial decrease in cell number reported subsequently, (7 and 14 days) and no cells detected 21 days after transplantation [48]. As survival was shown in *rag2*$^{-/-}$ mice for 3 weeks and reported for up to 5 weeks, the authors concluded that neonatal desensitisation was not successful using this cell type. In a subsequent publication, this group also reported the failure of neonatal desensitisation to promote survival of HUCB-NSC beyond 21 days following transplantation into infarcted rats [16]. In this case, however, poor graft survival was also found in control hosts treated with either CsA or a triple immunosuppression regime (CsA, azathioprine and methylprednisolone) [16]. Thus, this study does not conclusively demonstrate ineffectiveness of neonatal desensitisation, but may be simply indicative of a problem with survival of this donor cell type, or survival within this lesion model in this specific strain of rat. This highlights the need for adequate demonstration of survival of human donor cells in the immunocompetent adult host brain (e.g. immunosuppressed adult rats of the same strain), in order to define whether the failed graft is a result of immune rejection or that the cells have failed to survive for some other reason.

Neonatal desensitisation of mouse hosts

Although successful neonatal desensitisation of rat hosts has been demonstrated on numerous occasions, the same has not been shown in mice. Our initial studies were all carried out in rats, as we have more commonly used these for transplant hosts in order to take advantage of a wide range of behavioural tests optimised in rat models of PD and HD, and as we have regularly achieved decent survival of large within and between species transplants under appropriate immunosuppression. However, using mice as long term transplant hosts would allow the use of a wide range of available transgenic models that are not currently available in rats. In addition to models of neurodegeneration, a number of mouse models exist with modified immune systems, which would provide tools to investigate the mechanisms underlying neonatal desensitisation to human xenografts.

To date we have carried out a number of experiments to investigate the possibility of successful neonatal desensitisation of mouse hosts with little success, which have been reported in more detail elsewhere [18]. Initial experiments were carried out using the standard neonatal desensitisation protocol optimised previously for rat hosts [49] in CD-1 mice. However, in addition to poor survival of hPF(ctx) in the desensitised mouse hosts, there was also poor survival in immunosuppressed (daily CsA treated) mice [18] suggesting that there is a general problem of neural graft survival in the mouse. Again the use of outbred mice is of additional concern here, with a likely increase in the variability of transplant survival. Attempts to improve graft survival were made through modifications to the transplant protocol. These included transplanting an increased number of donor cells ($5x10^5$ vs $3x10^5$), and transplantation at different time-points following a quinolinic acid lesion, or into the intact striatum, in order to avoid the delivery of cells into a compromised environment. No improvement in survival was seen in any of these conditions. Varying the number of cells injected neonatally to improve neonatal desensitisation also proved unsuccessful [18]. The fact that survival in desensitised mice hosts was comparable to untreated hosts (~15% for both),

suggests that desensitisation was not successful in mice. However, as only 50% of grafts in hosts receiving a conventional immunosuppressant (CsA) survived it is not possible to conclusively determine whether or not desensitisation or other factors are responsible for the poor survival [18].

These findings are in line with research from other groups, which has also shown poor translation of neonatal desensitisation to mice using various host strains and donor cell types [37, 48]. Mattis et al performed a systematic investigation of neonatal desensitisation in three different strains of mice (both inbred and outbred) using three human stem cell types [37]. Both transgenic HD and wild type (WT) mice received i.p. injections on P2-3 of hPF-derived neural progenitor cells or ESC-derived NPCs and subsequently received bilateral striatal transplants of the same cell type at 5-10 weeks of age. Rejection of both cell types was seen at 6 weeks post-transplantation with dense infiltration of activated microglia and a lack of donor cells at the transplant site [37]. Treatment with CsA in desensitised and non-desensitised hosts was found to improve survival, although a reduction in the size of the transplants and the presence of microglial inflammation was still observed. Similar results were obtained using an inbred mouse strain (C57/BL6) with a neonatal injection of hESC-derived NPC at P5 followed by a transplant of the same cells at 2 months. No grafts were found to survive in untreated or desensitised hosts at 2 or 6 weeks post-transplantation [37].

Along with their experiments on desensitisation in rats, Janowski et al also tested whether mouse hosts (BALB/c) could be successfully desensitised to luciferase expressing human glial-restricted precursor (hGRP) cells derived from foetal brain [48]. The use of this line allowed detection of injected cells for both desensitisation and transplantation with *in vivo* bioluminescence imaging (BLI). The authors found a rapid reduction on BLI signal within a few days following desensitisation (<5 days) suggestive of elimination of peripherally injected cells. Following neural transplantation a reduction in BLI signal was again observed and was undetectable after 2 weeks in adult desensitised and naïve BALB/c mouse hosts. Transplants of hGRP cells delivered to adult *rag2*$^{-/-}$ hosts (n = 5), were found to survive well up to 3 weeks post-transplantation, however longer time-points are

necessary to determine whether these cells would survive long term. Dense infiltration of CD45+ immune cells was found in both desensitised and naïve hosts following sacrifice at 2 weeks post transplantation suggesting the loss of transplanted cells was due to rejection through the host immune system [48].

The rapid signal loss observed following neonatal i.p. injections in this study is interesting [48]. Although we do not know the mechanisms by which desensitisation of rats is produced, we may assume that the presence of donor cells for presentation to the immature neonatal immune system during development is necessary, and that this may not be possible if cells are rapidly rejected following neonatal injection. Support for this notion comes from Mattis et al's experiments; in which neonatal transplants of luciferase expressing human iPSC-derived neural precursor cells (iPSC-NPCs) to the neonatal mouse brain declined from approximately 9 days post transplantation with no cells remaining by 28 days [37]. These results were subsequently confirmed with histological analysis. The survival of the same cells was confirmed *in vitro* for several weeks, showing these cells to be healthy, and survival was also confirmed in neonatal immunodeficient (NOD SCID) mice. Additionally, transplants of hFNPs were also rejected in the neonatal mouse striatum, with increased immunoreactivity to the microglial marker Iba1 [37]. These findings differ to neonatal rat neural transplants of human cells, which have been consistently shown to survive long term [24, 25, 31, 36]. The findings of these two studies therefore suggest that, in contrast to rat hosts, xenogeneic donor cells are rapidly rejected in both the CNS and the periphery of neonatal mice. This may indicate that the immune system develops earlier in the postnatal period than in rats and is more developed by birth, or that it is simply more readily equipped for the rejection of human donor cells.

Xenograft rejection; a difference between rat and mouse hosts.

Numerous studies have investigated the rat host immune response to neural xenografts of mouse [50], porcine [51, 52] and human tissue. Some studies have been carried out to characterise the rejection of porcine tissue transplants in mice [17, 53, 54], however less work has been conducted specifically investigating the mouse host response to transplanted human cells. Thus, much neural transplant work has assumed a similarity between mice and rats as transplant hosts. In comparing the rat and mouse host immune response to transplants of porcine cells, Larsson et al found a much faster, more severe response from the mouse hosts [17]. We have recently compared the survival of human donor cells (hCTX) transplanted unilaterally into the striatum of QA lesioned rat (SD) and mouse (CD-1) hosts receiving no immunosuppression or daily CsA (Roberton, VH; unpublished data). At 10 days post-transplantation all grafts remained present in both CsA treated and untreated rat hosts. However rejection in mouse hosts was much more rapid, with only 50% of transplants remaining in both the untreated hosts and those receiving daily injections of CsA after 10 days. Findings discussed here regarding human to mouse xenografts are in line with this, showing a rapid clearance of human donor cells both in the periphery and the CNS [18, 37, 48]. The use of neonatal hosts does not avoid this, with cells injected intraperitoneally being cleared by around 5 days [48], and striatal transplants reducing in size from 9 days showing complete rejection by 21-28 days. In all cases dense infiltration of activated microglia is observed [18, 37]. It has been reported that higher doses of CsA may be required to successfully promote survival of xenografts in mouse hosts, although care is needed regarding the side effects [17]. Indeed, in all mouse experiments we have conducted, a dose of 10mg/kg daily via i.p. injection has been administered as we have found this to be successful in rat hosts [18]. Interestingly, Mattis et al reported survival of xenografts in CsA treated mouse hosts, albeit with some reduction in graft size up to 6 weeks post-transplantation. They administered 15mg/kg i.p daily, thus this may explain differences observed in survival between these studies [37]. Ultimately until issues with inconsistent xenograft survival in mouse hosts and the mechanisms behind successful neonatal desensitisation can be resolved it cannot be determined

whether the neonatal desensitisation protocol can be modified for successful application in mouse hosts.

Conclusions and future perspective

To date, neonatal desensitisation has been reliably demonstrated to promote survival of a range of human cell types in various rat models whilst avoiding the limitations associated with other immunosuppressant methods. It allows xenograft survival to a comparable extent to conventional immunosuppressant drugs, but for substantially longer [15, 45-47]. Desensitisation of rat hosts will therefore permit long term studies of graft maturation and functional outcomes that have not previously been possible. The method has not been successfully translated into mouse hosts, and the investigation of desensitisation in the mouse has revealed a number of fundamental differences between mice and rats in terms of the immune response to neural xenografts in both neonates and adults [18, 37, 48]. It will be important to unravel the mechanism underlying neonatal desensitisation, both to understand whether it can be modified for use in the mouse and to facilitate optimisation of the method in rats.

Executive summary

Why is long term assessment of human neural grafts in rodent hosts needed?

- Current immunosuppression methods are not adequate for full assessment of graft maturation and the measurement of functional improvements following transplantation.

Neonatal desensitisation as a method for assessing xenografts in the CNS

- Neonatal desensitisation takes advantage of the immaturity of the immune system of neonatal rats, promoting the long term survival of subsequent human xenografts in adult rat hosts.

Neonatal desensitisation in the rat host

- Effective desensitisation of rat hosts has been achieved in a range of models for as long as 40 weeks after transplantation.

- Poor xenograft survival in desensitised rat hosts may be related to, therefore adequate survival in immunosuppressed hosts must be demonstrated.

Neonatal desensitisation of mouse hosts

- Successful desensitisation of mouse hosts has not yet been demonstrated, and further study is needed to resolve the variability in xenograft survival in mouse hosts.

Cells used for neonatal desensitisation	Cells used for subsequent adult graft	Time post-transplant (weeks)	Number of grafts surviving (%)
mPF(WGE)	mPF(WGE)	10	11/15 (73%)
mFNP	mFNP	10	11/13 (85%)
hPF(ctx)	hPF(ctx)	40	48/55 (87%)
hPF(WGE)	hPF(WGE)	25	10/15 (66%)
hFNP	hFNP	12	10/13 (77%)
hES-N	hES-N	12	9/12 (75%)
hFNP	hPF(ctx)	12	11/13 (85%)
hPF(ctx)	hFNP	12	11/14 (79%)
hLiver	hPF(ctx)	12	8/13 (62%)
hPF(ctx)	hPFWGE	10	19/23 (83%)
None	hPF(ctx)	12	0/9 (0%)
None	hPF(WGE)	2	0/7 (0%)
None, but given CsA	hPF(WGE)	12	7/9 (77%)
None, but given CsA	hFNP	12	6/8 (75%)

Table 1 Summary of data in Kelly *et al.* (2009) [14] to show survival of human xenografts in adulthood where the neonate has been injected with cells from a similar (concordant) or different (discordant) origin. Transplant hosts in all experimental groups were SD rats. Graft survival is comparable to that seen in CsA-treated hosts and no graft survival is seen in host receiving no immune-modulatory treatment. *h=human, m=mouse, PF(ctx) = primary foetal cortex, PF(WGE) = whole ganglionic eminence, FNP = foetal neural precursors derived from foetal cortical tissue, hES-N = human ESChLiver = human liver, CsA = Cyclosporine A*

Strain	Cells used for desensitisation	Sample used for desensitisation	Lesion	Tissue Transplanted	Sample used for adult graft	Surviving (%)	Time post TX
SD	hPF(ctx)	A	QA	hPF(ctx)	C	3/7 (42.86%)	6 weeks
SD	hPF(ctx)	A	QA	hPF(ctx)	C	1/3 (33.33%)	9 weeks
SD	hPF(ctx)	A	QA	hPF(ctx)	D	4/4 (100.00%)	9 weeks
SD	CsA	-	QA	hPF(ctx)	C	2/2 (100.00%)	6 weeks
LH	hPF(ctx)	A	QA	hPF(ctx)	C	1/3 (33.33%)	6 weeks
LH	hPF(ctx)	A	QA	hPF(ctx)	D	3/3 (100.00%)	9 weeks
LH	CsA	-	QA	hPF(ctx)	C	3/4 (75.00%)	6 weeks
LH	CsA	-	QA	hPF(ctx)	D	3/3 (100.00%)	9 weeks
SD	hPF(ctx)	B	QA	hPF(ctx)	E	3/8 (37.50%)	6 weeks
SD	CsA	-	QA	hPF(ctx)	E	2/2 (100.00%)	6 weeks

Table 2 Previously unpublished data using neonatal desensitisation of rat hosts to promote survival of hPF(ctx) transplants. All hosts were desensitised with $1x10^5$ cells injected i.p. and subsequently transplanted unilaterally with $5x10^5$ cells. *SD = Sprague Dawley; LH = Lister Hooded;* hPF(ctx) *= human primary foetal cortical cells; hVM = human primary foetal ventral mesencephalon; QA = Quinolinic acid lesion; 6-OHDA = 6-hydroxydopamine lesion; TX = Transplant*

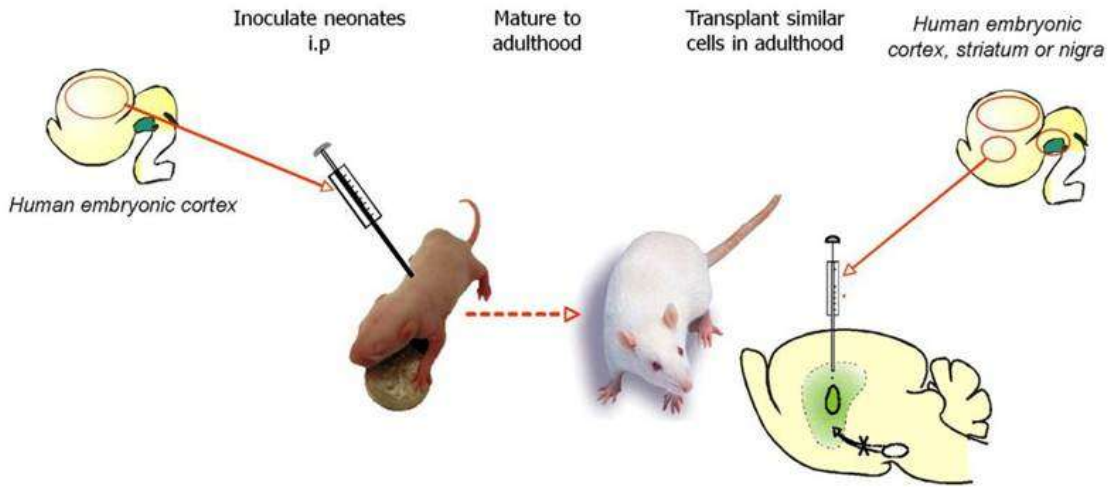

Figure 1 The neonatal desensitisation protocol. hPF(ctx) was used to inoculate early neonatal rat pups by i.p. injection. The animals were allowed to survive to maturity and then received a neural transplant of similar human PF(ctx) cells from a separate fetal sample. From Kelly *et al* (2009).

References

1. Brundin P, Nilsson OG, Strecker RE, Lindvall O, Astedt B, Bjorklund A: Behavioural effects of human fetal dopamine neurons grafted in a rat model of Parkinson's disease. *Exp Brain Res* 65(1), 235-240 (1986).

2. Dunnett SB, Nathwani F, Björklund A: *Chapter 16 The integration and function of striatal grafts*. In: *Progress in Brain Research*, S.B. Dunnett AB (Ed.^(Eds). Elsevier, 345-380 (2000).

3. Nakao N, Itakura T: Fetal tissue transplants in animal models of Huntington's disease: the effects on damaged neuronal circuitry and behavioral deficits. *Progress in Neurobiology* 61(3), 313-338 (2000).

4. Rosser AE, Bachoud-Lévi A-C: Clinical trials of neural transplantation in Huntington's disease. *Progress in brain research* 200, 345-371 (2012).

*** Reviews outcomes of clinical trials for transplantation in Huntington's Disease**

5. Evans JR, Mason SL, Barker RA: Current status of clinical trials of neural transplantation in Parkinson's disease. *Progress in brain research* 200, 169-198 (2012).

*** Reviews outcomes of clinical trials for transplantation in Parkinson's Disease**

6. Rosser AE, Dunnett SB: *Neural transplantation in Huntington's Disease*. In: *Cellular transplantation: From laboratory to clinic*, Transplantation DEBT-C (Ed.^(Eds). Academic Press, Burlington 417-437 (2007).

7. Barker RA, Mason SL, Harrower TP *et al.*: The long-term safety and efficacy of bilateral transplantation of human fetal striatal tissue in patients with mild to moderate Huntington's disease. *Journal of Neurology, Neurosurgery & Psychiatry*, (2013).

8. Hurelbrink CB, Armstrong RJ, Barker RA, Dunnett SB, Rosser AE: Hibernated human fetal striatal tissue: successful transplantation in a rat model of Huntington's disease. *Cell Transplantation* 9(6), 743-749 (2000).

9. Björklund A, Lindvall O: Cell replacement therapies for central nervous system disorders. *Nature neuroscience* 3, 537-544 (2000).

10. Kelly CM, Precious SV, Torres EM *et al.*: Medical Terminations of Pregnancy: A Viable Source of Tissue for Cell Replacement Therapy for Neurodegenerative Disorders. *Cell Transplantation* 20(4), 503-513 (2011).

11. Kim SU, De Vellis J: Stem cell-based cell therapy in neurological diseases: a review. *Journal of neuroscience research* 87, 2183-2200 (2009).

12. Precious SV, Rosser AE: Producing striatal phenotypes for transplantation in Huntington's disease. *Experimental Biology and Medicine* 237(4), 343-351 (2012).

13. Galea I, Bechmann I, Perry VH: What is immune privilege (not)? *Trends in immunology* 28(1), 12-18 (2007).

14. Barker RA, Ratcliffe E, Mclaughlin M, Richards A, Dunnett SB: A role for complement in the rejection of porcine ventral mesencephalic xenografts in a rat model of Parkinson's disease. *Journal of Neuroscience* 20, 3415 (2000).

15. Kelly CM, Precious SV, Scherf C *et al.*: Neonatal desensitization allows long-term survival of neural xenotransplants without immunosuppression. *Nature Methods* 6, 271-273 (2009).

**** First publication reporting successful neonatal desensitisation of rat hosts**

16. Jablonska A, Janowski M, Lukomska B: Different methods of immunosuppresion do not prolong the survival of human cord blood-derived neural stem cells transplanted into focal brain-injured immunocompetent rats. *Acta Neurobiol Exp (Wars)* 73(1), 88-101 (2013).

17. Larsson LC, Frielingsdorf H, Mirza B *et al.*: Porcine neural xenografts in rats and mice: donor tissue development and characteristics of rejection. *Experimental neurology* 172, 100-114 (2001).

18. Roberton VH, Evans AE, Harrison DJ *et al.*: Is the adult mouse striatum a hostile host for neural transplant survival? *Neuroreport* 24(18), 1010-1015 (2013).

*** Human to mouse striatal transplants and neonatal desensitisation**

19. Al Nimer F, Wennersten AA, Holmin S, Meijer X, Wahlberg L, Mathiesen T: MHC expression after human neural stem cell transplantation to brain contused rats. *NeuroReport* 15, 1871-1875 (2004).

20. Bertani T, Perico N, Abbate M, Battaglia C, Remuzzi G: Renal injury induced by long-term administration of cyclosporin A to rats. *The American journal of pathology* 127, 569-579 (1987).

21. Grasbon-Frodl E, Nakao N, Lindvall O, Brundin P: Phenotypic development of the human embryonic striatal primordium: a study of cultured and grafted neurons from the lateral and medial ganglionic eminences. *Neuroscience* 73(1), 171-183 (1996).

22. Grasbon-Frodl EM, Nakao N, Lindvall O, Brundin P: Developmental Features of Human Striatal Tissue Transplanted in a Rat Model of Huntington's Disease. *Neurobiology of disease* 3, 299-311 (1997).

23. Pearl JI, Lee AS, Leveson-Gower DB *et al.*: Short-term immunosuppression promotes engraftment of embryonic and induced pluripotent stem cells. *Cell stem cell* 8, 309-317 (2011).

24. Hurelbrink CB, Armstrong RJ, Dunnett SB, Rosser AE, Barker RA: Neural cells from primary human striatal xenografts migrate extensively in the adult rat CNS. *The European journal of neuroscience* 15(7), 1255-1266 (2002).

25. Hurelbrink CB, Barker RA: Migration of cells from primary transplants of allo- and xenografted foetal striatal tissue in the adult rat brain. *The European journal of neuroscience* 21, 1503-1510 (2005).

26. Nasonkin I, Mahairaki V, Xu L *et al.*: Long-term, stable differentiation of human embryonic stem cell-derived neural precursors grafted into the adult mammalian neostriatum. *Stem Cells* 27(10), 2414-2426 (2009).

27. Aubry L, Bugi A, Lefort N, Rousseau F, Peschanski M, Perrier AL: Striatal progenitors derived from human ES cells mature into DARPP32 neurons in vitro and in quinolinic acid-lesioned rats. *Proceedings of the National Academy of Sciences* 105(43), 16707-16712 (2008).

28. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE: RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68(5), 869-877 (1992).

29. Shinkai Y, Rathbun G, Lam K-P *et al.*: RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68(5), 855-867 (1992).

30. Kingsley CI, Nadig SN, Wood KJ: Transplantation tolerance: lessons from experimental rodent models. *Transplant international : official journal of the European Society for Organ Transplantation* 20, 828-841 (2007).

31. Lund RD, Rao K, Hankin MH, Kunz HW, Gill TJ: Transplantation of retina and visual cortex to rat brains of different ages. Maturation, connection patterns, and immunological consequences. *Annals of the New York Academy of Sciences* 495, 227-241 (1987).

32. Englund U, Fricker-Gates RA, Lundberg C, Björklund A, Wictorin K: Transplantation of Human Neural Progenitor Cells into the Neonatal Rat Brain: Extensive Migration and Differentiation with Long-Distance Axonal Projections. *Experimental Neurology* 173(1), 1-21 (2002).

33. Pollack IF, Lund RD: The blood-brain barrier protects foreign antigens in the brain from immune attack. *Experimental Neurology* 108(2), 114-121 (1990).

34. Kallur T, Darsalia V, Lindvall O, Kokaia Z: Human fetal cortical and striatal neural stem cells generate region-specific neurons in vitro and differentiate extensively to neurons after intrastriatal transplantation in neonatal rats. *Journal of Neuroscience Research* 84(8), 1630-1644 (2006).

35. Kallur T, Farr TD, Bohm-Sturm P, Kokaia Z, Hoehn M: Spatio-temporal dynamics, differentiation and viability of human neural stem cells after implantation into neonatal rat brain. *The European journal of neuroscience* 34(3), 382-393 (2011).

36. Zietlow R, Precious SV, Kelly CM, Dunnett SB, Rosser AE: Long-term expansion of human foetal neural progenitors leads to reduced graft viability in the neonatal rat brain. *Experimental Neurology* 235(2), 563-573 (2012).

37. Mattis VB, Wakeman DR, Tom C *et al.*: Neonatal immune-tolerance in mice does not prevent xenograft rejection. *Experimental Neurology* 254, 90-98 (2014).

**** Systematic investigation of neonatal desensitisation in various mouse strains and human to neonatal mouse neural transplants**

38. Billingham RE, Brent L: Acquired Tolerance of Foreign Cells in Newborn Animals. *Proceedings of the Royal Society of London. Series B - Biological Sciences* 146(922), 78-90 (1956).

39. Billingham RE, Brent L, Medawar PB: Actively acquired tolerance of foreign cells. *Nature* 172(4379), 603-606 (1953).

*** Neonatal transplant tolerance**

40. Adkins B, Jones M, Bu Y, Levy RB: Neonatal tolerance revisited again: specific CTL priming in mouse neonates exposed to small numbers of semi- or fully allogeneic spleen cells. *European journal of immunology* 34(7), 1901-1909 (2004).

41. Ridge J, Fuchs E, Matzinger P: Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science* 271, 1723-1726 (1996).

42. West LJ, Morris PJ, Wood K: Fetal liver haematopoietic cells and tolerance to organ allografts. *The Lancet* 343(8890), 148-149 (1994).

43. Modigliani Y, Burlen-Defranoux O, Bandeira A, Coutinho A: Neonatal Tolerance to Alloantigens is Induced by Enriched Antigen-Presenting Cells. *Scandinavian journal of immunology* 46(2), 117-121 (1997).

44. Bandeira A, Coutinho A, Carnaud C, Jacquemart F, Forni L: Transplantation tolerance correlates with high levels of T- and B-lymphocyte activity. *Proceedings of the National Academy of Sciences* 86(1), 272-276 (1989).

45. Zhang S, Jiang YZ, Zhang W *et al.*: Neonatal desensitization supports long-term survival and functional integration of human embryonic stem cell-derived mesenchymal stem cells in rat joint cartilage without immunosuppression. *Stem Cells Dev* 22(1), 90-101 (2013).

46. Singhal S, Bhatia B, Jayaram H *et al.*: Human Müller Glia with Stem Cell Characteristics Differentiate into Retinal Ganglion Cell (RGC) Precursors In Vitro and Partially Restore RGC Function In Vivo Following Transplantation. *Stem Cells Translational Medicine* 1(3), 188-199 (2012).

47. Jayaram H, Jones MF, Eastlake K *et al.*: Transplantation of Photoreceptors Derived From Human Müller Glia Restore Rod Function in the P23H Rat. *Stem Cells Translational Medicine* 3(3), 323-333 (2014).

48. Janowski M, Jablonska A, Kozlowska H *et al.*: Neonatal desensitization does not universally prevent xenograft rejection. *Nat Meth* 9(9), 856-858 (2012).

49. Kelly CM: Desensitisation of neonatal rat pups for xenotransplantation with human tissues. (2009).

50. Finsen BR, Sørensen T, Castellano B, Pedersen EB, Zimmer J: Leukocyte infiltration and glial reactions in xenografts of mouse brain tissue undergoing rejection in the adult rat brain. A light and electron microscopical immunocytochemical study. *Journal of neuroimmunology* 32, 159-183 (1991).

51. Armstrong RJE, Harrower TP, Hurelbrink CB *et al.*: Porcine neural xenografts in the immunocompetent rat: immune response following grafting of expanded neural precursor cells. *Neuroscience* 106(1), 201-216 (2001).

52. Larsson LC, Czech KA, Brundin P, Widner H: Intrastriatal ventral mesencephalic xenografts of porcine tissue in rats: immune responses and functional effects. *Cell Transplant* 9(2), 261-272 (2000).

53. Larsson LC, Anderson P, Widner H, Korsgren O: Enhanced Survival of Porcine Neural Xenografts in Mice Lacking CD1d1, But No Effect of NK1.1 Depletion. *Cell Transplantation* 10(3), 295-304 (2001).

54. Larsson LC, Corbascio M, Widner H, Pearson TC, Larsen CP, Ekberg H: Simultaneous inhibition of B7 and LFA-1 signaling prevents rejection of discordant neural xenografts in mice lacking CD40L. *Xenotransplantation* 9(1), 68-76 (2002).