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# Neonatal immune-tolerance in mice does not prevent xenograft rejection

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## Abstract

Assessing the efficacy of human stem cell transplantation in rodent models is complicated by the significant immune rejection that occurs. Two recent reports have shown conflicting results using neonatal tolerance to xenografts in rats. Here we extend this approach to mice and assess whether neonatal tolerance can prevent the rapid rejection of xenografts. In three strains of neonatal immune-intact mice, using two different brain transplant regimes and three independent stem cell types, we conclusively show that there is rapid rejection of the implanted cells. We also address specific challenges associated with the generation of humanized mouse models of disease.

### Keywords

Neonatal tolerance; neonatal immunity; xenograft; Huntington's disease; immune rejection

## Introduction

Human induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) are proving informative for *in vitro* and *in vivo* modeling of human diseases (Mattis and Svendsen, 2011) and provide promise for cell-based transplantation treatments. While *in vitro* iPSC models are useful, an *in vivo* "humanized" chimeric animal model of disease via transplantation of diseased human iPSC-derived cells could provide a better model for understanding disease mechanisms and therapeutic screening. This is especially true when evaluating the functional effects of stem cell engraftment into disease-related transgenic mutants. Human iPSC-derived neurons or ESCs injected into the mouse or non-human primate striatum are able to survive and make connections (Kriks et al., 2011; Maria et al., 2013). However, one of the major challenges for the field is appropriate immune



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suppression in these xenograft models. Immunosuppression is not always effective for xenografts, is often cost-prohibitive for long-term studies, especially in larger animals, and has also been shown to ameliorate some neurological diseases (Rosenstock et al., 2011), thereby confounding experimental results.

To avoid rejection issues in adult transplants, neonatal immune-tolerance, which takes advantage of the under-developed immune system of neonatal mammals by introducing a foreign substance (i.e., cells) soon after birth so that it will be recognized as “self” later in life, has been used in several studies. Human neural progenitor cells (hNPCs) injected into neonatal rodents survive without suppression and integrate into the entire neurological axis (Windrem et al., 2004; Windrem et al., 2008). In theory, human iPSC-derived neural tissue or ESCs could also be transplanted into neonatal animals to generate humanized models without the need for continual suppression. While there are numerous studies injecting human cells into both neonatal and adult rats (Denham et al., 2012; Englund et al., 2002; Jablonska et al., 2010; Kallur et al., 2006; Kopen et al., 1999; Lundberg et al., 2002; Rachubinski et al., 2012; Windrem et al., 2004), there are far fewer that have used neonatal or adult mice (Windrem et al., 2004; Windrem et al., 2008).

Neonatal desensitization is a new strategy for long-term immune protection of human neural cells transplanted into the adult brain, without the need for immunosuppression (Kelly et al., 2009; Peiguo et al., 2012; Zhang et al., 2013). Rodents are given intraperitoneal (i.p.) injections of the donor cells within a few days after birth, and receive transplants of the same cells into the brain several months later. In one study, 62–87% of Sprague-Dawley rats had demonstrable graft survival of mouse or human fetal- or ESC-derived NPCs 10–40 weeks later (Kelly et al., 2009). However, when this experiment was repeated in BALB/c mice or Wistar rats the transplanted cells survived less than two weeks (Janowski et al., 2012). These data strongly suggest that there may be a differing potential for neonatal or adult acceptance of transplants or desensitization between species, or even between background strains, of rodents.

In this current set of studies, we compared multiple techniques in specific mouse strains and utilized several stem cell types to examine tolerance of the neonatal and adult mouse brain to neural xenografts. We show that in contrast to rat neonates, mouse neonates and adult mice are uniquely sensitive to human neural xenografts derived from iPSCs, ESCs or fetal NPCs. In our report, and with multiple mouse strains used, injections in neonatal mice or prior sensitization did not reduce the severe rejection of transplanted cells. In addition, *in vivo* luciferase imaging proved to be a powerful predictor of graft survival in the striatum, although it was susceptible to false negatives. Together these studies show that neonatal and adult mice reject human cells and that, in this context, immune tolerance techniques are not sufficient to prevent this rejection.

## Methods

### Cell Culture for Neonatal Striatal Transplants

Non-integrating iPSCs were grown as previously described (Ebert et al., 2009; The_HD_iPSC_Consortium, 2012). Briefly described, iPSC colonies were gently scraped off of matrigel coated plates after 5 minutes of accutase treatment. Colonies were then pelleted in a conical tube (1000 RPM, 5 min) and resuspended in a neural progenitor media containing DMEM:F12 media with 2%B27 without vitamin A (Life Technologies 12587-010), 1% Pen-Strep-Amphotericin (PSA), 100ng/ml epidermal growth factor (EGF, Peprotech AF-100-15) and 100 ng/ml fibroblast growth factor (FGF2, Peprotech 100-18B). Cells were then grown in suspension as spheres in a poly-HEMA coated flask for up to 30 passages. To provide stable luciferase expression for future *in vivo* detection, spheres were

dissociated into a single-cell suspension using trypLE (Life Technologies 12604-013) and LUC2-lentivirus particles (100ng p24/ml of media) were added for 24 hours before adding fresh media. Cells then reformed spheres in suspension, which were expanded for several passages before *in vitro* validation of luciferase expression. Prior to transplantation, cells were dissociated with trypLE, plated on matrigel-coated dishes, and allowed to differentiate in DMEM:F12 with 1%N2 (Life Technologies 17502-048) and 1%PSA for ~0–40 days. For *in vivo* transplantation, iPSC-derived cells were dissociated prior to transplantation with trypLE into a single cell suspension, treated with DNase for 5 minutes, washed and resuspended (50,000 cells/μl) in conditioned growth media. Methods to grow hNPCs from fetal cortex (G010 cell line) have been described (Svendsen et al., 1997; Svendsen et al., 1998) and conform to National Institute of Health and Cedars-Sinai Medical Center guidelines for collection of fetal tissues. Fetal–derived NPC neurospheres were prepared for transplantation as previously described (Klein et al., 2005; Suzuki et al., 2007). Briefly, neurospheres were dissociated into a single cell suspension with trypLE, treated with DNAse for 5 minutes, washed and resuspended to 50,000 cells/μl in DMEM media.

### Neonatal Striatal Stem Cell Injections

B6C3F1/J or NOD SCID (NOD.CB17-Prkdcscid/J) (Jackson labs) mice were bred, housed and had surgeries/imaging performed under institutional review board (IRB/SCRO) and institutional animal care and use committee (IACUC) approval. Neonates were transplanted into the striatum on postnatal day 1–4. Prior to injection, hypothermic anesthesia was induced by placing pups on frozen gel packs for 1–3 minutes, until cessation of movement (Passini and Wolfe, 2001). Bilateral stem cell striatal injections were performed one time between p1–p5 using a 10 μl Hamilton syringe (Hamilton, Reno, NV) fitted with a finely drawn beveled glass capillary needle (aluminosilicate (sutter: SCHOTT 8252) with a 1mm outer diameter; after pulling, the needle has an inner diameter of 100μm and a 40% bevel). The injections were performed directly through the skin and skull using a stereotaxic frame with the coordinates 1.5 mm rostral of the transverse sinus, 1.5 mm lateral of the superior sagittal sinus, and 3mm in depth from the skin as guidelines to approximate the location of the striatum. 1 μl of cells (50,000 cell/μl) in solution were bilaterally injected. At this early postnatal stage the skull is not fully calcified and therefore easily punctured with the beveled glass capillary needle. Upon completion of surgery, mice were warmed on a heating pad until movement resumed and returned to littermates in a warmed receptacle containing nestlet material from the home cage. Pups were observed for at least 30 minutes to ensure safe recovery, “re-scented” with nest material, and returned to the dam and littermates.

### Neonatal desensitization and adult transplantation in transgenic HD and WT mice

Wild-type (B6C3F1/J) and Huntington’s disease (HD) (B6C3F1/J-TgN(HD82Gln)81Dbo(N171)) transgenic mice carrying a mutant *Huntingtin* gene were purchased from Jackson Labs and bred to produce litters for desensitization as previously described (Ramaswamy et al., 2009). Human fetal NPCs (hfNPC) (G010 cell line) were derived as neurospheres from early gestation cortex and fully characterized as previously described (McBride et al., 2004; Svendsen et al., 1998) and later expanded *in vitro* for three months using a three-dimensional adherent cell culture method (Wakeman et al., 2009). Cultures were synchronized for optimal transplantation in relation to the timing of live birth for initiation of the desensitization protocol. In addition to hfNPCs, embryonic stem cell-derived NPCs (cell line STEMEZ™ hNP1™, Aruna Biomedical) were cultured according to manufacturer’s instructions. Both hfNPC and hNP1 cells were dissociated into single cells with Accutase and resuspended in conditioned media for injection. Transgenic HD and littermate WT neonatal (P2–P3) pups under hypothermic anesthesia (Wakeman et al., 2009) received a single i.p. injection of $1.5\times10^5$ cells/ μl hfNPC (G010) or hNP1 in 2μl conditioned medium (total $3\times10^5$ cells) using a heat drawn glass micropipette. Animals were

then warmed to reverse anesthesia, and returned to the dam in their home cage. After 3 weeks, all mice were genotyped by PCR, randomized, and assigned to receive bilateral striatal stereotaxic injections as previously described (Ramaswamy et al., 2009). Adult mice were injected pre-symptomatically or post-symptomatically for HD (AP: 0.5 ML: +/− 1.80, and DV −2.85 relative to Bregma) with 2μl control media, hfNPC, or hNP1 ($1\times10^5$ cells/ul in 2μl control media per hemisphere; $2\times10^5$ cells total/hemisphere) that were phenotypically identical to those used for desensitization at birth. A subset of these mice were given daily i.p. injections of 15mg/kg cyclosporine (Bedford Labs) (Supplementary Table S1). All experiments on HD and WT littermate mice were performed in accordance with the guide for the care and use of laboratory animals under approval from Rush University Medical Center IACUC.

### Neonatal desensitization and adult transplantation in transgenic C57BL6 and FVB mice

ENStem-A Human Neural Progenitor cells (Millipore, Massachusetts) were cultured using manufacturer's procedures then electroporated using the AMAXA Nucleofector system (Lonza, Basel, Switzerland) with a plasmid expressing green fluorescent protein (GFP) driven by the CAGG promoter for expression in human stem cells (Liew et al., 2007). For stable transfection selection, the cells were subjected to 1μg/ml puromycin (Sigma-Aldrich). For implantation into mice, cultured ENStem-A-GFP cells were trypsinized, centrifuged, resuspended in HBSS with FGF-2 (20 ng/mL) and counted. 50,000 cells per animal (1 μl) were injected i.p. into P5 C57Bl6 or FVB pups, while two other pups received vehicle alone. Two months later, animals anesthetized with isofluorane received bilateral striatal injections (AP: 0.00 ML: +/− 2.00, and DV −3.25 relative to Bregma) of 100,000 GFP-NPCs (2 μl / injection at an injection rate of 0.5 μl /min) or vehicle (HBSS with 20 ng/ml EGF) using a 5 ul Hamilton microsyringe (33-gauge). Wounds were sealed using bone wax on the skull and closed with dermabond. Mice were placed on heating pads until fully recovered from anesthesia and then housed individually to protect the wound from littermates. One month after re-implantation mice were sacrificed. Animal experiments were under approval from the UC Irvine IACUC.

### Live animal imaging for in vivo transplant survival detection

Starting three days post-transplantation, animals were imaged at Cedars-Sinai Medical Center imaging core facility. Bioluminescence imaging procedure was performed using a Xenogen Spectrum *In Vivo* Imaging System (IVIS). Pups were subcutaneously injected with 150 mg/kg luciferin (VivoGlo™, Promega) 10 minutes prior to imaging to allow for circulation of the luciferin substrate throughout the pup. Pups were then imaged in the Xenogen IVIS for 5 minutes to achieve maximum sensitivity, under manual restraint (under 7 days) or isoflurane (over 7 days). Images were then subsequently analyzed to quantify cerebral luciferase expression (subtracting the background expression from the body of the animal).

### Cell and Tissue Staining

*In vitro* cells were plated onto laminin/ polyornithine coated coverslips for 3 hours and then fixed with 4% paraformaldehyde (PFA). They were then stained with mouse anti-Nestin (Millipore MAB5326, 1:500) and goat anti-luciferase (Promega G7451, 1:500). Animals were transcardially perfused with saline followed by 4% PFA. The brain was removed and post-fixed in 4% PFA overnight, then placed into 20% sucrose until they sunk followed by 30% sucrose overnight before sectioning on a microtome (35 microns). Brain tissue was then processed by immunohistochemistry (Ramaswamy et al., 2009). Briefly described, free-floating sections were blocked in 10% donkey serum and stained with goat anti-luciferase, SC101 (Stem Cells Inc. AB-101-U-050, 1:500), SC121 (Stem Cells Inc. AB-121-

U-050, 1:500), human nuclei (huNuc; Chemicon MAB1281 1:200), or rabbit anti-Iba1 (Wako 019-19741, 1:500) overnight. Sections were then stained with appropriate fluorescently-conjugated antibodies and nuclei were stained with DAPI. Counts for human nuclei (sc101) were performed in 35 micron (μm) thick sections, 1/6th apart in a total of 12 sections per animal (35microns × 12 = 420microns). Counting criteria included co-localization of sc101 labeling with the nuclear marker DAPI. Due to the small nature of the grafts and lack of cell migration, systemic sampling (entire graft in each section) was performed using a 20X magnification image (1–5 image per section), captured with a Leica AF3500 microscope and analyzed using metamorph software. As human nuclei size was < 10 μm and each section counted was spaced at a distance 210 μm, no correction was performed on cell counts as selected 1/6th sampling eliminated the chance of repeat counting the same marker. To estimate the total number of cells in each graft, the number of cells counted was multiplied by 6 (sampling series interval). Some (Supplementary Table S1) adult desensitized and cyclosporine treated WT and HD (B6C3F1/J) mice were also stained using the DAB method. These sections were blocked with 3% serum of the secondary antibody species and 3% bovine serum albumin then stained with primary antibody. Sections were washed and stained with a biotinylated secondary antibody followed by the ABC and DAB reaction as previously described (Ramaswamy et al., 2009).

## Results

### Injections of human iPSC-derived neural progenitors into the mouse striatum are rejected within four weeks

The under-developed immune system of neonatal rats has been utilized so that allografts and xenografts transplanted within the first few days of life are recognized as “self” and hence not rejected (Englund et al., 2002; Kallur et al., 2006; Kopen et al., 1999; Lundberg et al., 2002; Streilein, 1979; Windrem et al., 2008). This technique allows for transplantation without the need for immunosuppression. To establish whether this may hold true for mice, the striata of neonatal B6C3F1/J mice were bilaterally transplanted with a total of 100,000 (50,000 cells per hemisphere; LUC2; Supplementary Fig. 1A–D, Supplementary Table S1) luciferase over-expressing iPSC-derived neural progenitor cells (iPSC-NPCs). Luciferase-expressing cells were chosen for transplant in order to perform *in vivo* imaging on the same animals over multiple time points to longitudinally track transplant survival. Mice were therefore imaged weekly for luciferase expression starting ~3 days after transplant until sacrifice. Over this time, there was a clear reduction in luciferase expression (Fig. 1A). This loss of expression was most likely due to transplant rejection, but other imaging limitations such as animal growth (increased head size, calcification of the skull, etc) that could potentially physically obstruct the machinery’s ability to detect the luciferase signal cannot be discounted.

To address the reason for the observed luciferase reduction and if it correlated with cell survival, animals were sacrificed at time points over 35 days and graft survival was quantified by counting all detected human nuclei (SC101) in one in six serial sections of the brain. At the early time point (day 7), transplants targeted to the striatum showed adequate survival with many cells having a neuronal morphology and staining with an antibody specific to luciferase (transplanted iPSC-NPCs) (Fig. 1B,C). Many cells appeared to migrate along white matter tracks (data not shown). However, in accordance with the luciferase expression data, graft survival declined dramatically over time such that by day 9 only ~6,000 cells were still remaining (~6% of initial graft; (Fig. 1D)). Cell survival continued to decline to ~3,000 by day 10 (~3%), ~100 by day 21 (1%) and there were no detectable cells at day 28 or after (Fig. 1D; Supplementary Table S1). To exclude the possibility that cells were compromised during the transplantation procedure, the cells remaining post-transplant

were plated onto matrigel-coated coverslips. These cells survived for several weeks in culture (data not shown), suggesting that cells were healthy at the time of transplantation and could survive well beyond day 28 *in vitro*.

Since a decrease in luciferase expression *in vivo* corresponded to a decrease in iPSC-NPC survival, we next tested whether the luciferase expression directly correlated to surviving cell number in the graft. Therefore, luciferase expression one to two days prior to sacrifice was plotted against the number of iPSC-NPCs estimated in a graft post-sacrifice (Fig. 1D). While 19/21 animals had a direct Gaussian distribution ($R^2$ value 0.996) of luciferase expression to cell number, two animals had low expression but very high cell counts. Therefore, one cannot state that luciferase expression is an accurate quantitative measure of *in vivo* cell survival. Positive or negative luciferase expression determined by image analysis can be used as a qualitative indicator, as 20/21 animals were correctly identified as having graft survival (above 500 cells remaining) or rejection (less than 250 cells) (Fig. 1E,F). The misidentification of one animal that was identified as having rejected a transplant could be due to an inadequate injection of the luciferin, highlighting the importance of accurate delivery and repeated animal analysis. It is important to note that while there was one false-negative, there were no false-positives (Fig. 1G). Overall, the reduction in luciferase expression is a reliable qualitative measure of the reduction in graft survival seen over time in the neonatally-transplanted animals.

## Neither human iPSC-derived nor fetal-derived neural progenitor transplants survive in the mouse striatum due to host rejection, and the result was not be ameliorated by immunosuppression via maternal lactation

From results above, iPSC-derived neural progenitors were rapidly rejected in neonatally-transplanted immune competent mice and raise the possibilities that the iPSC-derived neural progenitors were uniquely sensitive to rejection or selectively mediate a robust immune response in the mice. To address whether these iPSC-derived neural progenitors were uniquely sensitive to rejection in the mice, we next tested transplantation of hfNPCs, which have been successfully transplanted into both neonatal and adult rodents by multiple laboratories (Capowski et al., 2007; Englund et al., 2002; Klein et al., 2005; Svendsen et al., 1997; Svendsen et al., 1998). Following neonatal striatal injections of hfNPCs, using identical methods to those described for iPSC-NPCs, mice were sacrificed at key time points to determine graft survival. Much like the decline observed with the transplantation of the iPSC-NPCs, by day 9/10 only ~7,000 hfNPCs (~14%) were remaining, and by day 21 (or 28) no hfNPC graft survival was detected (Fig. 2A). It was therefore concluded that rejection of xenografts in neonatal mice occurs irrespective of the iPSC or fetal source of neural progenitors.

To address whether this extensive graft reduction could be due to an acute immune rejection from infiltrating microglia and macrophages we examined the expression of Iba1 in mice transplanted with human iPSC- or fetal-derived NPCs. Increased expression of Iba1 was seen within all the transplanted areas (detected by SC101 and luciferase), compared to sham-transplanted (media) animals with much lower Iba1 levels (Fig. 2B). This result suggested that iPSC-NPC grafts were not surviving due to an immunologic response mounted by the wildtype B6C3F1/J mouse strain over time. To confirm this, human iPSC-derived NPCs were transplanted into neonatal immunodeficient (NOD SCID) mice (Tamaki et al., 2002; Uchida et al., 2000). In contrast to the complete transplant rejection in B6C3F1/J mice by 28 days, transplants into NOD SCID mice survived well up to 62 days post-transplantation using live animal imaging (luciferase) (Fig. 1A). Post mortem analysis confirmed this survival and even suggested *in vivo* cell proliferation (Fig. 1D). Therefore it

can be concluded that human xenografts are indeed capable of surviving in the mouse brain, but only in an immunodeficient environment.

Given that both iPSC-NPC and hfNPC transplants were rejected in the neonatal immune competent mice and that injection of immunosuppressive agents is not possible in neonates, we postulated that immunosuppression given to the dam mouse might be delivered to the pups. Support for this idea comes from human studies indicating that FK506 (tacrolimus/prograf) is excreted during lactation, and that infants would thereby receive approximately 0.2% of the dam's weight-adjusted dose (French et al., 2003). Therefore, in an attempt to enhance transplant survival in this model, we administered 3 mg/kg to the lactating mouse from birth, on the assumption that the pups would receive approximately 0.6 mg/kg. Analysis of cell grafts at day 9/10 post-transplant showed no significant difference between pups with or without FK506 immunosuppression (Figure 2C). Furthermore, FK506 given to the dams did not rescue long-term graft survival, as no detectable cells were seen on or after 35 days post-transplant. We did not notice a decreased rejection in response to immunosuppression via maternal lactation delivery, although we cannot be sure that sufficient FK506 reached the pups to be effective.

### Desensitization in the neonatal mouse does not result in survival of human ESC or fetal–derived neural progenitors transplanted in the adult brain

A study to assess neonatal desensitization demonstrated that a neonatal tolerance protocol of injecting fetal cells i.p. into rat neonates would promote the survival of a subsequent transplant of the same cells into the adult brain (Kelly et al., 2009). Given the lack of survival of either fetal- or iPSC-derived hNPCs transplanted into the neonatal mouse striatum, we next asked whether we could use neonatal tolerance in mice to promote survival of an adult transplant. Specifically, transgenic HD and WT (B6C3F1/J) mice were desensitized on postnatal day 2–3 by an i.p. injection of human fetal-derived NPCs (hfNPCs) or ESC-derived NPCs (STEMEZ-hNP1; hNP1) and then adult mice at either 5 (pre-symptomatic HD) or 10 (post-symptomatic HD) weeks of age received bilateral striatal transplants of the same cell type that they had received as neonates in order to determine if the xenograft could survive in desensitized mice and if transplanting into the diseased brain would affect the survival and integration of the cells (Supplementary Table S1).

Animals that were neonatally desensitized followed by an adult striatal transplant were sacrificed at 16-weeks old to analyze the brain tissue for graft survival and integrity (Ramaswamy et al., 2009). Human cytoplasmic marker (huCyto) and Iba1 staining showed that there was marked immunological rejection at the host-graft interface under all conditions; ie. desensitized, non-immunosuppressed WT (B6C3F1/J) and HD mice transplanted with hfNPCs pre- or post-symptomatically (Fig. 3A–H; Supplementary Fig. S2A–H) or human ESC-derived NPCs grafted post-symptomatically (hNP1; Supplementary Fig. S3). Iba1 staining revealed dense populations of activated microglia at the host-graft interface, infiltrating the injection site devoid of hfNPCs. Pre-symptomatically treated animals appeared to have a decrease in huCyto and Iba1 at the host-graft interface. Animals grafted with hfNPCs or ESC-derived hNPCs post-symptomatically displayed marked signs of ongoing host rejection at the host-graft interface at 6-weeks post-transplantation. Both WT and HD mice showed graft rejection, suggesting that combining a diseased environment, which is possibly conducive to cell survival, with sensitization is still insufficient to promote graft survival. Desensitized animals mock-transplanted with control medium displayed little to no increase in Iba1 expression at the injection site indicating that the localized increase in immunological response was due primarily to rejection of grafted cells, not trauma from the injection (data not shown).

Given that long-term cell survival has been undetectable in all preceding transplant paradigms, desensitized and non-desensitized WT and HD (B6C3F1/J) mice were cyclosporine-treated before bilateral transplantation into pre- or post-symptomatic animals to ensure that hfNPCs could indeed survive with appropriate suppression (Supplementary Table S1). At 10 days and 6 weeks post-transplantation, huCyto, human-specific nuclei, and human-specific nestin staining showed survival of hfNPCs in the striatum (Fig. 3I–X) (Supplementary Fig. S2I–X and S4). Graft size was considerably reduced at 6 weeks compared to 10 days post-transplantation, indicating some cell death over time, possibly due to incomplete cyclosporine immunosuppression and ensuing host immunological rejection evidenced by a similar Iba1 host-graft interface pattern (Fig. 3I–X, Supplementary Fig. S2). However, in general, at six weeks post-transplantation, grafted hfNPCs that survived, appeared healthy, projected huCyto fibers into the peripheral host tissue, and were morphologically characteristic of differentiated neural cells suggesting normal maturation of the cells in cyclosporine-treated immmunosuppressed HD (Fig. 3I–X) and WT mice (Supplementary Fig. S2I–X).

The (B6C3F1/J) mouse strain used to this point is an outbred line. To determine if mouse genetic background strains had an effect on the apparent lack of desensitization, the FVB and C57/BL6 inbred mouse strains (Supplementary Table S1) were desensitized at postnatal day 5 with human ESC-derived NPCs (ENStem-A-GFP) and were transplanted at 2 months of age with the same cells. Animals sacrificed at either two or six weeks post-transplantation had no detectable grafts by GFP, huCyto or SC101 staining (data not shown), irrespective of whether or not they had been initially desensitized.

These results strongly suggest that neonatal desensitization with hfNPCs or human ESC-derived NPCs (hNP1 or ENStem-A-GFP) in B6C3F1/J (WT or transgenic HD), C57/BL6, or FVB mice does not render animals immunologically privileged to an adult striatal cell graft. Therefore, the novel desensitization technique first reported by Kelly et al. in rats (Kelly et al., 2009) is not supported by these data, indicating species and strain variability likely has a significant impact on the outcome and successful use of the “desensitization” methodology in both neonatal and adult mice.

## Discussion

A number of *in vitro* models of neurodegenerative diseases using iPSCs have been generated and show great promise for disease modeling and drug screening. However, these models lack the vascular and immune systems that may be needed to achieve fully differentiated cells, In contrast, transplantation of cells into live animals could permit more extensive cell maturation and differentiation and provide an understanding of how they function *in vivo* (Svendsen, 2013). Unfortunately, the rapid rejection of xenografts is a major challenge for generating chimeric disease models or for testing the therapeutic potential of human stem cell transplants. Neonates may circumvent this complication with human xenografts as the immature rodent immune system can be desensitized to develop these models without the need for immune suppression[1,12]. In addition, the developing neonatal brain may provide an appropriate environment for maturing the neural progenitor cells.

The study by Kelly et al (Kelly et al., 2009) demonstrated that neonatal desensitization in Sprague-Dawley rats allows for xenogenic transplantation without the need for immunosuppression. However, a follow-up study using hfNPCs in BALB/c mice or human umbilical cord-derived NPCs in Wistar rats (Janowski et al., 2012), found neither cell type surviving after 21 days in either species. Cells did, however, survive in a rag2−/− immunodeficient mouse, indicating that they were capable of surviving in immunoprivileged animals (Janowski et al., 2012). These authors postulated that the reason for the success of

the desensitization technique for xenotransplantation in the Kelly et al. study was the specific cell type used. We propose that it is not in fact due to the cell line, but most likely due to the strain and species of rodent used.

Interestingly, most neonatal transplants to date have been performed in Sprague-Dawley rats, even though transgenic diseased mice may be a more relevant model for researchers studying a particular disease. The only successful mouse neonatal xenotransplantation (Windrem et al., 2004) was in an inbred shiverer model. Follow-up studies were however all performed in shiverer mice crossed with an immunodeficient rag 2 knockout mice (shi+/+; rag2−/−) (Sim et al., 2011; Wang et al., 2013; Windrem et al., 2008). To our knowledge, no other studies of neonatal transplants in an outbred mouse line with an intact immune system have been reported. While there is some evidence of tolerance in fetal rodent transplantations (Carletti et al., 2004; Marcus et al., 2008; Muotri et al., 2005), it has also been found that the maternal immune system may limit engraftment potential (Nijagal et al., 2011). We show conclusively in this report that human xenografts at the doses tested are not accepted in outbred (B6C3F1/J) neonatal or sensitized adult mice but rather are rapidly rejected, in contrast to previous results in neonatal Sprague-Dawley rats (Englund et al., 2002).

Immune suppression of neonates is not an alternative as daily injections are not feasible and we unfortunately show immunosuppression through the milk of lactating dams is insufficient to protect neonatal grafts. This lack of immune protection and ensuing graft rejection in outbred neonates was reiterated with neonatal sensitization and subsequent transplantation of human iPSC-, hESC-, and fetal-derived NPCs into adults, both in outbred and inbred strains. The host immunogenic response could not be overcome by transplanting hfNPCs, a robust cell source shown here and previously to survive in immune-suppressed animals, nor by transplanting into a diseased brain, shown previously to provide a more conducive environment for cell survival. We have previously shown that very high doses of xenografted cells will be rejected even in immune-suppressed rats where lower doses of cells survive in the same groups of animals (Ostenfeld et al., 2000). Thus, it is possible that lower doses of cells may not be rejected in the models used here, which we are currently testing. However, this result would also reduce the practicality of this technology as our previous experience in immune deficient mice shows 100,000 cells is the minimum number required to produce robust grafts for functional outcomes. A manuscript has been recently published highlighting the rejection issues associated with both allografts and xenografts in mice (Roberton et al., 2013), even with immune suppression. CD-1 strain mice were given striatal transplants with allografts or human xenografts, using either cyclosporine or neonatal tolerization to suppress an immune response, and allowed to mature for 6–12 weeks. This publication reports that 0–20% of the human grafts into neonatally desensitized mice survived for this time period and the authors suggest that the mouse brain may have a more hostile microglial response to grafting. The differing mouse strain may account for the small but greatly varied percentage graft survival seen in Roberton et al, as opposed to no survival seen in this study.

Animal-human chimera disease models need to be assessed for cell integration and cell function. As all animals in this study immunologically rejected the transplants, it was not surprising that HD animals (pre- or post-symptomatically treated with either hfNPC or hNP1) displayed no significant behavioral improvement in functional motor skills (hind-limb clasping and rotorod tests, data not shown) and no extended survival time (data not shown, Supplementary Table S1) compared to WT littermates. Cell survival and integration may be enhanced by lesions (Behrstock et al., 2008) and functional integration has been seen in the adult lesioned non-human primate brain (Emborg et al., 2013), however, in the current study the HD disease state did not appear to enhance survival. Clearly, robust and

consistent transplantation/immunosuppression protocols must be developed so that animal-human chimera models can be used widely across laboratories to generate reliable results.

### Conclusions

In conclusion we have shown that there is severe immune-rejection after transplantation of human iPSC-, hESC- or fetal-derived neural progenitors into the neonatal mouse brain and that neonatal desensitization methods are not sufficient to promote subsequent graft survival in adults across multiple strains of mice.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **iPSCs** | induced pluripotent stem cells |
| **ESCs** | embryonic stem cells |
| **NPCs** | neural progenitor cells |
| **HD** | Huntington’s disease |
| **i.p** | intraperitoneal |

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## Highlights

- Immune-rejection after xenotransplantation into the neonatal mouse brain
- Neonatal mouse desensitization does not promote subsequent adult graft survival
- Immune-rejection is independent of neural progenitor source (ESC, iPSC, fetal)
- Combined, this data points to the absence of murine neonatal immunity induction

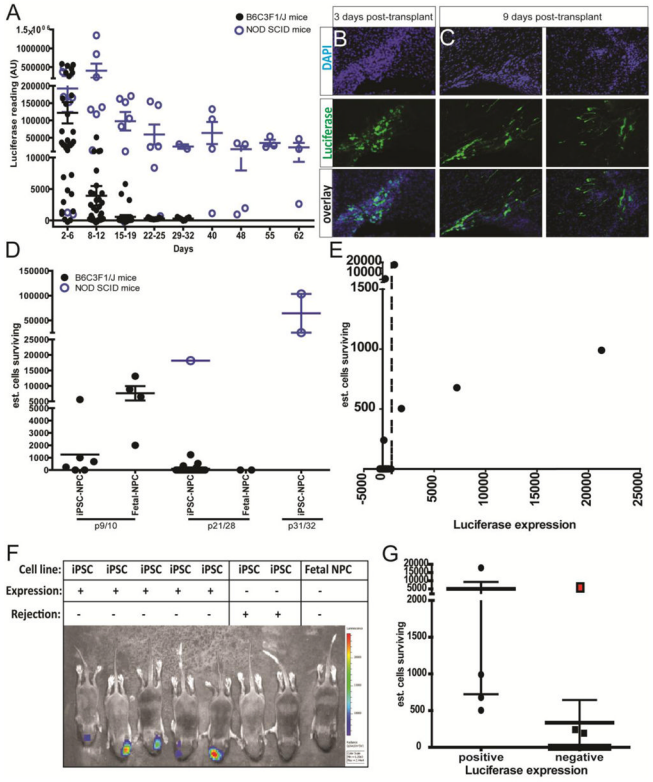


**Fig. 1.**

*In vivo* detection of neonatal striatal injections of stem cells is high initially but decreases over time, which is qualitatively related to cell transplant survival. (A) Neonatal B6C3F1/J pups who received striatal injections of iPSC-derived neural cells lose bioluminescence in 22–25 days, as opposed to NOD SCID mice who maintain expression for 62 days. Mice (B6C3F1/J or NOD SCID) were given an injection of luciferin and imaged weekly from ~day 2–6 (1–5 days post-transplant) until day 62 and bioluminescence (average radiance [p/s/cm$^2$/sr]) above background was measured. Individual animals were plotted by image and bar represents the mean (error bars represent SEM). (B) Morphology of cells 3 days post-transplant. (C) Example images of morphology and increased neurite outgrowth of luciferase-expressing cells 9 days post-transplant. (D) Neonatal B6C3F1/J striatal injections of stem cells survive initially but decrease over 21–28 days, while NOD SCID mice have equal to, or more, human cells detected than initially transplanted at that time. Of the initial 100,000 cells transplanted, by day nine ~6% are remaining in the B6C3F1/J pups. The estimated number of surviving cells decreases over time so that by 28 days no live cells were found by immunohistochemistry for SC101 (human nuclear marker). (E) Luciferase expression (bioluminescence (average radiance [p/s/cm$^2$/sr])) of animals prior to sacrifice plotted against estimated cell number of transplant remaining. 19 of 21 animals plotted have a Gaussian distribution. However, 2 of 21 animals have high cell counts but low expression. Dotted vertical line represents “negative” imaging levels (no cells found surviving). (F) Representative image of luciferase expression in pups demonstrating qualitative transplant survival. iPSCs transplanted had luciferase expression but fetal NPCs did not (negative control). (G) Only one animal was misidentified as “rejected” by imaging that had cells (indicated in red). If the outlier is rejected (Q-test 90% confidence) then there is a significant difference of $p<0.0267$ by student’s t-test between the groups.

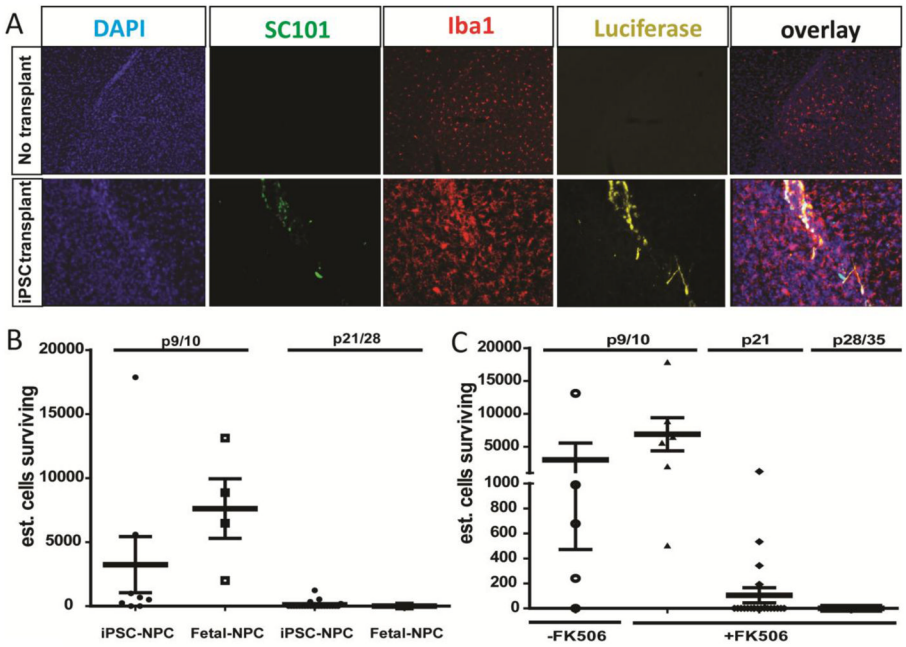


**Fig. 2.**

Both iPSC-derived and fetal-derived NPCs are rejected by the host immune system and immunosuppression via maternal lactation does not ameliorate this rejection. (A) Iba1 stain for infiltrating macrophages and microglia at p10 demonstrates increased immunogenicity in graft area. Iba1 is upregulated and has activated morphology as compared to the same area in a non-transplanted animal. SC101 and luciferase (cytoplasmic) indicate grafted cells. (B) No significant difference in graft survival was seen between neonatal mice transplanted with iPSC-NPC or fetal NPCs at either early or late time points (one-way ANOVA). (C) Dams were given immunosuppression (3 mg/kg/d FK506) for the first 21 days post-whelping. No significant difference was seen in estimated cell survival with or without FK506 at day 9/10 (one-way ANOVA). Additionally, FK506 did not rescue cell transplant survival at later time points (21/28 or 35 days).

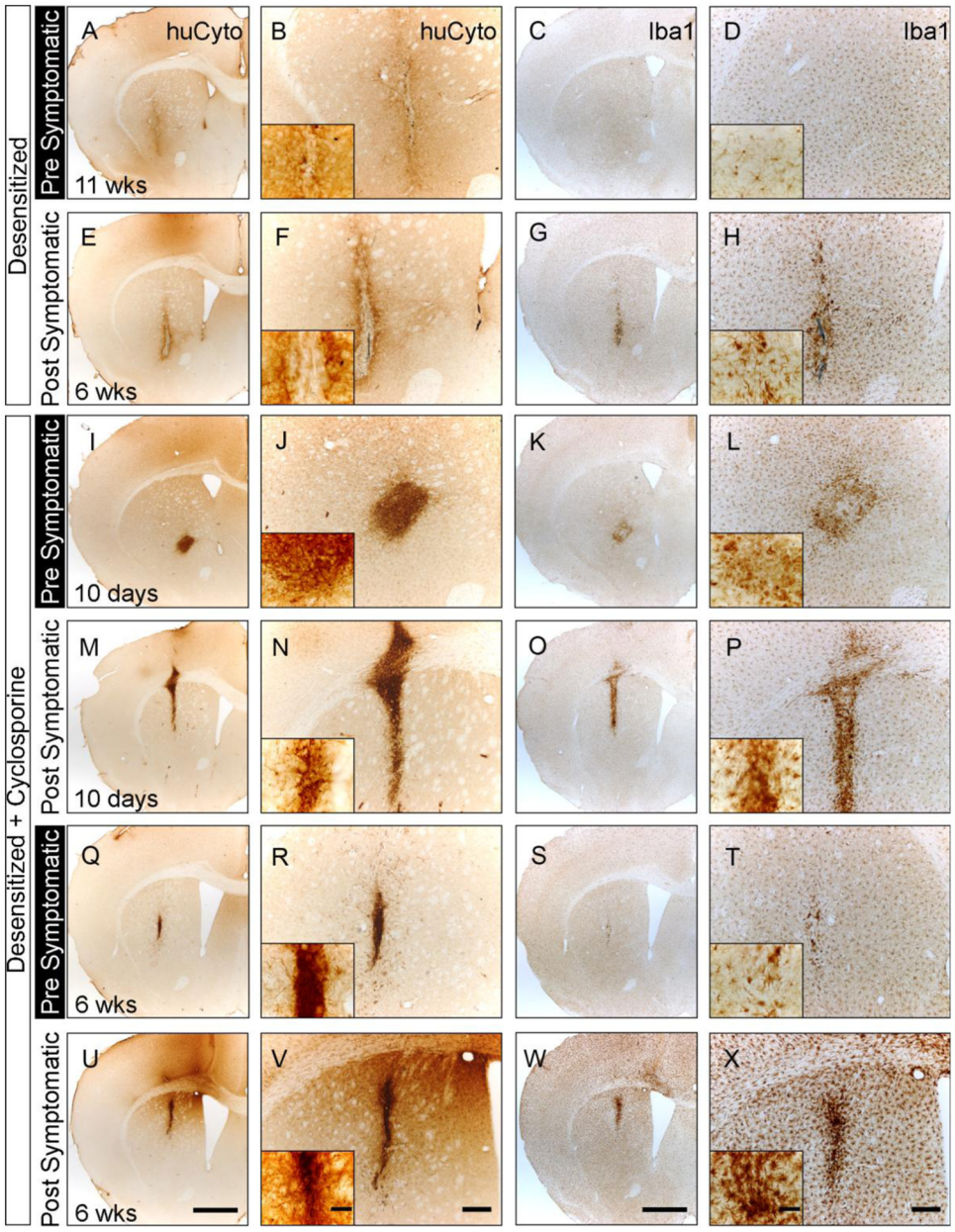


**Fig. 3.**

Grafted hfNPC are rejected in desensitized HD (B6C3F1/J) mice. Immunohistochemical staining in desensitized HD mice treated without cyclosporine (A–H) or with cyclosporine (I–X) revealed marked graft immunological rejection of huCyto hfNPC grafts at both pre-symptomatic (A–D, I–L, Q–T) and post-symptomatic (E–H, M–P, U–X) time points. The host-graft interface at the site of injection was demarcated by increased levels of Iba1 microglia with activated morphology. (scale bar = 1mm A, C, E, G, I, K, M, O, Q, S, U, W) (scale bar = 200μm B, D, F, H, J, L, N, P, R, T, V, X). Insets are higher magnification of graft-host interface (scale bar= 25μm).