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Direct *In Vivo* Assessment of Human Stem Cell Graft-Host Neural Circuits

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Abstract

Despite the potential of stem cell-derived neural transplants for treating intractable neurological diseases, the global effects of a transplant's electrical activity on host circuitry have never been measured directly, preventing the systematic optimization of such therapies. Here, we overcome this problem by combining optogenetics, stem cell biology, and neuroimaging to directly map stem cell-driven neural circuit formation *in vivo*. We engineered human induced pluripotent stem

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Author Contributions

B.B. planned stem cell transplantation experiments, derived the iPSC line, transfected cells, prepared the transplantations, obtained *in vitro* confocal images, and wrote sections of the paper. H.J.L. conducted the transplantations, took care of animals including cyclosporine injections, and conducted the *in vivo* electrophysiology. J.L. conducted the transplantations, ofMRI experiments, image reconstruction, and brain perfusions. A.J.W. performed ofMRI data analysis, acquired confocal images of brain slices, and wrote the paper. P.L. assisted in stem cell transplantation, immunohistochemistry, and animal care. P.Z. assisted in stem cell culture, FACS, transfection, differentiation, and immunocytochemistry. A.S. conducted the *in vitro* patch clamping. R.D. supervised A.S. and provided required resources for *in vitro* patch clamping. R.R.P. supervised B.B. and P.Z. and provided required resources for stem cell culture and derivations. J.H.L. designed and planned the study, analyzed and interpreted the data, wrote the paper, supervised all aspects of the experiments and personnel, and provided required resources.

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cells (iPSCs) to express channelrhodopsin-2 and transplanted resulting neurons to striatum of rats. To non-invasively visualize the function of newly formed circuits, we performed high-field functional magnetic resonance imaging (fMRI) during selective stimulation of transplanted cells. fMRI successfully detected local and remote neural activity, enabling the global graft-host neural circuit function to be assessed. These results demonstrate the potential of a novel neuroimaging-based platform that can be used to identify how a graft's electrical activity influences the brain network *in vivo*.

Keywords

functional MRI; optogenetics; stem cell; transplant

1. Introduction

Stem cell mediated therapies aim to directly restore the neural circuitry lost during disease progression of the central nervous system (CNS). Applications of interest include Parkinson's disease (Zeng and Couture, 2013), Alzheimer's disease (Lee et al., 2010a), amyotrophic lateral sclerosis (Dimos et al., 2008), retinal degenerative disease (Tucker et al., 2011), stroke (Lee et al., 2010c), and spinal cord injury (McDonald et al., 1999). Yet despite the potential of stem cell based CNS repair, successful therapy development has been hindered by the lack of methods that can assess the functional connections and causal interactions between a neural transplant and host circuitry in an intact animal. For example, recent efforts to characterize transplant integration include *in vivo* tracking of labeled stem cells (Hoehn et al., 2002; Jasmin et al., 2011; Modo et al., 2004), visualization and electrophysiological recordings of synapse formation (Benninger et al., 2003; Czupryn et al., 2011; Espuny-Camacho et al., 2013; Oki et al., 2012), and behavioral monitoring of the host organism (Ben-Hur et al., 2004; El-Akabawy et al., 2012; Hargus et al., 2010; Kriks et al., 2011; Parish et al., 2008). These techniques provide important information on stem cell survival and engraftment, but are limited by their inability to directly assess the whole-brain functional impact of the graft on host neural networks *in vivo*. The ability to selectively stimulate engrafted cells with optogenetic techniques provides a unique opportunity to interrogate the functional integration of neural grafts and identify functional graft-host synapses (Tonnesen et al., 2011; Weick et al., 2010; Weick et al., 2011). Here, we report a novel method that enables direct optogenetic stimulation of stem cell-derived human neurons, combined with whole-brain high-field fMRI, to directly evaluate the causal influence of a graft's electrical activity on the global brain network as it integrates into the nervous system of a living subject.

2. Materials and Methods

2.1 Human stem cell preparation

To maximize translational potential, we used a human induced pluripotent stem cell (iPSC) line (Huf6) previously shown to possess hallmark characteristics of pluripotency both *in vitro* and *in vivo* (Byers et al., 2011; Nguyen et al., 2011). To evaluate the generalizability of our methods, we also performed experiments with neurons derived from the H9 human

embryonic stem cell line (WiCell Research Institute). We engineered the cells to express the light-sensitive ion channel channelrhodopsin-2 (Boyden et al., 2005; Deisseroth et al., 2006; Nagel et al., 2005) (ChR2) prior to their transplantation in rats, which enabled selective, temporally precise control over the electrical activity of neural grafts *in vivo* (Fig. 1). Cells were transfected overnight using a concentrated EF1a-ChR2-EYFP lentivirus construct carrying the opsin (ChR2) and an enhanced yellow fluorescent protein (EYFP) reporter with the titer tightly controlled to ensure cell survival. The EF1a promoter was chosen since it can achieve long-term expression of transgenes in stem cells. Cells with high EYFP expression were selected manually or with fluorescence activated cell sorting (FACS) at one week post-transfection.

Both cell lines were differentiated following an optimized dual SMAD inhibition protocol based on Chambers et al (Chambers et al., 2009), for which the expression profiles of resulting neurons had previously been characterized by gene expression analysis, immunostaining, *in vitro* spontaneous differentiation, and *in vivo* teratoma assay (Nguyen et al., 2011). Briefly, pluripotent stem cells were manually plated on matrigel-coated plates and allowed to expand in iPSC media (mTeSR1, StemCell Tech) until ~30% confluency was reached. Media was then changed to 15% KSR DMEM/F12 and supplemented with Noggin and SB431542, a TGF- β small molecule inhibitor, to achieve dual SMAD inhibition. Neural rosettes were manually transferred to matrigel-covered wells with Sonic hedgehog (SHH) and fibroblast growth factor 8 (FGF8) patterning approximately 3–5 days after their appearance. Cells were then treated with SHH, FGF8, brain-derived neurotrophic factor (BDNF), and ascorbic acid to promote differentiation, and matured with TGF- β 3, BDNF, glial cell-derived neurotrophic factor (GDNF), dibutyl cyclic AMP, and ascorbic acid.

2.2 Immunocytochemistry

To characterize the expression of different cellular markers *in vitro*, cells were fixed in 4% paraformaldehyde/PBS for 15–30 min, washed twice with PBS, and blocked with 3% donkey serum in PBS for 1 hr at room temperature. For nuclear or intracellular staining, cells were permeabilized with 0.3% Triton-X100 for 30 min at room temperature. Samples were incubated in primary antibodies overnight at 4 °C, and then washed with PBS before fluorescent-conjugated secondary antibodies were added and incubated for 1 hr at room temperature. Finally, the cells were rinsed and counterstained with DAPI. Stained samples were imaged directly on a Zeiss confocal microscope or LEICA inverted fluorescent microscope. Primary antibodies and their dilutions were as follows: GFP (1:1000, Abcam), Tuj1 (1:200, Abcam), TH (1:500, Pel-Freez Biologicals), PITX3 (1:200, Abcam), nestin (1:100, Santa Cruz), and DCX (1:200, Santa Cruz). Secondary antibodies included Alexa Fluor 488 donkey anti-chicken (1:200, Jackson ImmunoResearch, 703-545-155), Alexa Fluor 594 donkey anti-mouse (1:200, Jackson ImmunoResearch, 715-585-150), and Alexa Fluor 594 donkey anti-rabbit (1:200, Jackson ImmunoResearch, 711-585-152).

2.3 In vitro electrophysiology

To confirm that derived neurons would be functional upon transplantation, current clamp recordings were performed on ChR2-EYFP-tagged cells at room temperature at days 30–50 post-differentiation. Briefly, neurons grown in 35 mm plastic dishes were visualized with a

40X air objective on an inverted Nikon microscope (Ellipse TE2000) and recorded using an EPC10 amplifier (HEKA, Germany) with pipettes made of borosilicate glass (BF150-110-10, Sutter Instruments, Novato, CA; 3–6 MOhm when filled with intracellular solution). The following solutions were used (in mM): 140 NaCl, 2.5 KCl, 2.5 CaCl₂, 2 MgCl₂, 1 NaH₂PO₄, 20 glucose, 10 HEPES, pH 7.4 (extracellular); 120 KGlu, 20 KCl, 4 NaCl, 4 Mg₂ATP, 0.3 NaGTP, 10 Na₂PCr, 0.5 EGTA, 10 HEPES, pH 7.25 (intracellular). Recordings were acquired using Patchmaster software (HEKA), filtered at 2 kHz, and digitized at 10 kHz. Fluorescent neurons were stimulated with 473–491 nm light using an X-cite light source.

2.4 Stem cell transplantation

Cells were transplanted to the striatum of female nude rats ($n = 15$, 5 weeks old, 150–200 g). Normal animals were used to avoid biases resulting from utilizing animal models of disease. The goal was to demonstrate the technology in an unbiased setting as a technology that can be generalized to any animal model. Animal husbandry and experimental manipulation were in strict accordance with National Institute of Health, UCLA Institutional Animal Care and Use Committee (IACUC), and Stanford University IACUC guidelines. Prior to transplantation, cells were dissociated with either TrypLE (Invitrogen), Collagenase IV (Life Technologies), or manually. Isolated cell colonies were selected and concentrated either by centrifugation and suspension, or by gravity and aspiration until the desired concentration (40K cells/uL on average) was reached. Prior to surgery, animals were anesthetized in a knockdown box using 5% isoflurane for induction. Isoflurane was then maintained at 2–3% for the remainder of the surgery. Buprenorphine (0.05 mg/kg) was injected subcutaneously to minimize post-operative discomfort. Heads were shaven and animals were secured onto a stereotaxic apparatus. Artificial tears were applied on the eyes to prevent them from drying out during surgery. Following a midline incision, betadine and 70% ethanol were applied on the bare scalp to minimize the possibility of infection. A small craniotomy was made using a dental drill above the target transplantation site in dorsal striatum (AP: +0.5 mm, ML: +3.0 mm right hemisphere, DV: –5.5 to –5.0 mm). Next, 3 μ L of stem cells were injected into dorsal striatum using a 10 μ L Hamilton syringe and 26 gauge metal needle (World Precision Instruments Inc.) at a 150 nl/min flow rate driven by a micro-syringe pump controller. Upon completion of the injection, the syringe needle was left in place for 10 additional minutes before being slowly withdrawn. A custom designed bare fiber-optic cannula (Dorics Lenses Inc.) was inserted into the craniotomy, mounted on the skull with the fiber optic end 0.2 mm above the transplant site, and secured using one layer of metabond (Parkell Inc). The incision was sealed by tissue glue. Animals were then kept on a heating pad until recovery from anesthesia. Cyclosporine (10 mg/kg) was injected daily after stem cell transplantation for 5 weeks and every other day for 2 additional weeks to prevent transplant rejection. Transplantations were randomly ordered between hESC and human iPSC to avoid any bias associated with varying experiment conditions that could not be easily controlled such as surgeon fatigue.

2.5 Optogenetic fMRI (ofMRI) experiment

fMRI scanning was performed in a 7 Tesla small animal dedicated MRI system at UCLA (Bruker Biospec) or Stanford University (Discovery MR901, Agilent Technologies)

approximately 50 to 300 days after transplantation using a previously described protocol (Lee et al., 2010b; Weitz et al., 2015). Briefly, animals were ventilated (45–55 strokes/min) under light anesthesia using a ventilator (Harvard Apparatus, Model 683 Small Animal Ventilator) and calibrated vaporizer with a mixture of O₂ (~35%), N₂O (~65%), and isoflurane (1–1.5%). An optical fiber of 62.5 μ m core diameter was connected to a 473 nm laser source from CrystaLaser (Reno, NV) and coupled with the implanted cannula. Light power was set to 2.5 mW at the fiber tip, which corresponds to 815 mW/mm² irradiance with a 62.5 μ m diameter fiber. Control experiments were performed with a 594 nm laser source from Laserglow Technologies (Toronto, ON) with the power adjusted to $2.5 \text{ mW} \times (594\text{nm}/473\text{nm}) = 3.1 \text{ mW}$ ($\sim 1 \text{ W/mm}^2$) to maintain the same energy delivery with yellow light as blue light. Expiratory CO₂ was kept at 3–4% using a capnograph (Harvard Apparatus, Type 340 for small rodents), and body temperature was maintained at 36.5–37.5 °C using heated airflow. The rat's eyes were covered with opaque tape and the ferrule was covered with black heat-shrink tubing in order to avoid visual stimulation.

A gradient recalled echo (GRE) BOLD sequence was used to acquire fMRI images during optical stimulation. The fMRI acquisition was designed to have 35×35 mm² in-plane field of view (FOV) and 0.5×0.5×0.5 mm³ spatial resolution with a sliding window reconstruction to update the image every repetition time (TR) (Fang and Lee, 2013). The two-dimensional, multi-slice gradient-echo sequence used a four-interleave spiral readout (Glover and Lee, 1995; Kim et al., 2003), 750 ms TR, and 12 ms echo time, resulting in 23 imaging slices. The spiral k-space samples were reconstructed through a 2-dimensional gridding reconstruction method (Jackson et al., 1991). Finally, to ensure robust detection of the fMRI signal, fast and accurate motion correction was performed using a custom-designed GPU-based system (Fang and Lee, 2013). A single fMRI scan consisted of six 20 s periods of continuous light delivered once per minute over 6 min. Multiple scans were collected in series to create a single scanning session to improve the signal-to-noise ratio.

All fMRI data processing was performed using custom-written programs and mrVista (Stanford Vision and Imaging Science and Technology Laboratory, Stanford, CA) in Matlab (MathWorks, Inc., Natick, MA). Motion-corrected images belonging to scans of the same animal and scanning session were first averaged together. The average 4D images were then aligned to a common coordinate frame, using a six-degree-of-freedom rigid body transformation. Time series were calculated for each voxel by calculating the percent modulation of the BOLD signal relative to a 30 s baseline period collected prior to stimulation, followed by detrending to correct for scanner drift. Active voxels were identified as those whose time series were significantly synchronized to six cycles of stimulation using the quantity of coherence (Lee et al., 2010b). Briefly, coherence values were calculated using the discrete Fourier transform, defined as the time series' magnitude at the frequency of repeated stimulations (i.e. 1/60 Hz) divided by the sum-of-squares of all frequency components. An activation threshold of 0.35 coherence was used. Assuming Gaussian noise and ~470 degrees of freedom (computed with the SPM software environment), the Bonferroni-corrected *P*-value for this threshold can be estimated (Bandettini et al., 1993) to be less than 10^{−9}. For some remote areas of modulation (i.e. septal nuclei and cingulate cortex), a threshold of 0.25 was used due to lower functional signal-to-noise ratio. This corresponds to a corrected *P*-value less than 2×10^{-3} .

Representative activation maps in Fig. 4 were generated from the average of 4 scanning sessions of a single animal that were spatially smoothened using a 2 pixel full-width half maximum Gaussian kernel. The scanning sessions corresponding to each image included 7 to 9 individual six-stimulation cycle scans averaged together. Representative activation maps in Fig. S7 were generated as the average of 5 individual scans. A digital standard rat brain atlas (Paxinos and Watson, 2006) was used to localize activated voxels to specific brain regions.

Displayed time series were generated by first averaging the time series of active voxels from a selected ROI within each scanning session. The resulting time series were then averaged across scanning sessions of the same animal, and then across animals, so that each animal was weighted equally. Therefore, activity was detected within individual animals, and not a group average. Corresponding hemodynamic response functions (HRFs) were generated by averaging these time series over their six cycles of stimulation. The first data point of each HRF was also subtracted as a baseline offset to define the signal's relative percent modulation from the onset of stimulation. Scanning sessions that did not give rise to active voxels with positive BOLD in the reported ROIs were excluded from analysis.

2.6 In vivo electrophysiology

Following the completion of ofMRI studies (at least 350 days post-transplantation), *in vivo* electrophysiology recordings were performed to confirm the neuronal basis of BOLD signals observed during fMRI experiments. Recordings were collected with a 16-channel acute (non-chronically implanted) extracellular silicon probe electrode (model A1x16-100mm-100-177, 1–1.2 MΩ at 1 kHz, 50 μm diameter, NeuroNexus Inc., Ann Arbor, MI) during optical stimulation at the stem cell transplantation site while rats were anesthetized under 1.3% isoflurane. For recordings in striatum, a custom optrode was used that included the silicon probe electrode attached to a 62.5 μm diameter optical fiber. The probe tip was positioned approximately 0.4 mm deeper than the tip of the optical fiber to ensure that recorded neurons were sufficiently illuminated. For each remote (non-striatal) recording, a small craniotomy was performed above the desired coordinate to insert the extracellular electrode: hippocampus (AP: –4.6 mm, ML: –1.7 mm left hemisphere, DV: –3.5 mm), cingulate cortex (AP: 2.76 mm, ML: +0.5 mm right hemisphere, DV: –2.1 mm), retrosplenial cortex (AP: –6.7 mm, ML: –1.5 mm left hemisphere, DV: –1.9 mm). Electrophysiology signals were then measured while the stem cell transplantation site (striatum) was optically stimulated with the implanted optical fiber coupled to a 473 nm laser diode from CrystaLaser (Reno, NV). Power was set to 2.5 mW at the fiber optic tip (this corresponds to 815 mW/mm² irradiance with our 62.5 μm diameter fiber). Signals were acquired at 40 kHz and band-pass filtered between 150 and 8000 Hz. Neuronal spikes were recorded using the OmniPlex neural data acquisition system (Plexon Inc., Dallas, TX). Single units were discriminated offline based on principal component analysis via Offline Sorter (Plexon Inc., Dallas, TX). For each experiment, neuronal firing rates were compared between the pre-stimulation period and the stimulation and post-stimulation periods. All recordings were performed in animals used for functional imaging. Different animals were used for local and remote extracellular recordings.

2.7 Ex vivo evaluation of stem cell integration

To verify stem cell integration and evaluate cell fate, animals were anaesthetized with isoflurane and transcardially perfused using ice-cold 4% paraformaldehyde (PFA) in 0.01 M PBS. Brains were subsequently extracted and fixed in PFA overnight before being equilibrated in 10%, 20%, and then 30% sucrose in PBS at 4 °C. Coronal brain sections were cut in two series with thicknesses of 200 and 40 µm using a freezing microtome (Leica VT1000s) and stored in cryoprotectant (25% glycerol, 30% ethylene glycol, in PBS) at 4 °C until processed with standard immunohistochemistry procedures. Samples were then imaged with a Leica scanning laser microscope and oil immersion objectives. Primary antibodies and dilutions were as follows: NuMA (1:250, Abcam), NeuN (1:400, Millipore), YFP/GFP (1:500, Millipore; 1:1000, Aves), hNCAM (1:200, Santa Cruz Biotechnology), PSA-NCAM (1:200, Millipore), Tuj1 (1:2000, Covance), DCX (1:100, Santa Cruz Biotechnology), Ki67 (1:500, Abcam), nestin (1:500, R&D Systems), GFAP (1:1000, DAKO), and Iba1 (1:1000, Abcam). Secondary antibodies included 488 donkey anti-chicken (1:200–1:500), 594 donkey anti-mouse (1:200), Cy5 donkey anti-goat (1:500), Cy5 donkey anti-mouse (1:500), and Cy3 donkey anti-goat (1:400) from Jackson ImmunoResearch, and 555 donkey anti-rabbit (1:500) from Life Technologies. Animals were prepared for immunohistochemistry when their cannula became inadvertently removed and they had to be sacrificed to avoid infection and comply with the IACUC animal protocol.

3. Results

Virally mediated optogenetic techniques were used in these experiments to achieve targeted control over the activity of stem cell-derived neurons. Following transfection *in vitro*, ChR2-EYFP-expressing cells were enriched by fluorescence-activated cell sorting, resulting in robust ChR2 expression that was preserved after multiple rounds of passaging (Fig. 1C and Figs. S2A and B). After 22–23 days of differentiation, the iPSC line yielded cells that maintained high levels of ChR2 expression, presented morphology indicative of maturing neurons, and were positive for the neuron-specific beta-III tubulin marker Tuj1 (Fig. 1D). Cells also stained positive for the neuronal markers doublecortin (DCX), PITX3, and tyrosine hydroxylase (TH; Figs. S1A–C). A small number of cells expressed nestin, suggesting that some remained in an undifferentiated state (Fig. S1C). Similar expression patterns of ChR2, Tuj1, DCX, TH, and nestin were observed in the hESC line (Figs. S2C–E). To confirm that cells were optically excitable, we performed *in vitro* current clamp recordings during continuous illumination with 473 nm light. Light-evoked action potentials could be driven in ~40% of iPSC- and hESC-derived neurons, consistent with prior studies that used depolarizing current injections (Nguyen et al., 2011) (Figs. 1E and F and Fig. S2F; $n = 5/12$ tagged cells). All cells that did not generate action potentials exhibited a voltage deflection in response to light, suggesting that they were neurons not yet fully mature. These findings demonstrate that ChR2 was stably and functionally expressed in the stem cell-derived population prior to its transplantation.

We next transplanted the cells to striatum of severe combined immunodeficiency rats (Fig. 2A). Resulting iPSC-derived grafts spanned several millimeters in size over three months after transplantation (Fig. 2B). Approximately 85% of cells positive for the human-specific

nuclear antigen marker NuMA co-expressed ChR2 ($n = 1332/1572$ cells), while 98% of ChR2-positive cells co-expressed NuMA ($n = 1332/1354$ cells; Fig. 2C). Regarding cell fate, approximately 15% of ChR2-positive cells co-expressed the mature neuronal marker NeuN ($n = 61/402$, Fig. 2D). Grafts also stained positive for Tuj1, the neuronal migration protein doublecortin, and the neural cell adhesion molecules hNCAM and PSA-NCAM (Figs. 2E–G and I), suggesting that the engrafted non-NeuN-expressing cells were likely immature neurons. In agreement with this, expression of the neural stem cell marker nestin was mostly absent from grafts (Fig. 2H). Only a small number of cells (~1%) stained positive for Ki67, indicating that cells were not tumorigenic (Fig. 2I). With regard to host-graft acceptance, expression of the astrocytic marker GFAP was slightly elevated at the graft core – consistent with previous studies of intrastriatal transplants (Fricker et al., 1999) – while Iba1-expression (used to identify microglia) was not markedly increased in the vicinity of the graft (Fig. S3). For the hESC-line, ChR2-expressing cells were also found to survive at least 5 months after transplantation and stain positive for doublecortin (Figs. S4A and B). Like the iPSC-line, the hESC-derived population was mostly absent of Ki67 (Fig. S4B).

Confirming that engrafted cells integrated into the global brain circuit, ChR2-expressing axons projected beyond the graft via different white matter tracts, including the ipsilateral and contralateral corpus collosum, the cingulum, and the cerebral peduncle (Figs. 2B and 3). Axons were also observed in various grey matter regions such as the ventral pallidum, ventral striatum, septal nuclei, dorsal thalamus, and cingulate cortex. While these findings provide evidence for the structural integration of transplanted grafts, anatomical data alone are unable to assess whether and how these grafts functionally integrate into the local and remote networks.

To non-invasively visualize the functional integration of transplanted cells, we next performed selective stimulation (20 s at 473 nm) of transplanted cells during simultaneous high-field, whole-brain fMRI (termed ofMRI) *in vivo* (Fig. 4A). Visualizing transplanted cell-driven functional activity using fMRI is a highly challenging task given the relatively small number of neurons that are potentially active in these experiments. Therefore, to increase sensitivity of detection, we used a novel inverse gauss-newton motion-correction method (Fang and Lee, 2013). This highly accurate and fast motion correction method increases robustness of detection in the presence of the motion that inevitably occurs during experiments with live subjects. In addition, to enable visualization of neural activity modulated by transplanted cells – the location of which cannot be easily predicted – the whole brain was imaged at a high spatiotemporal resolution. With this novel imaging approach, activated voxels, identified as those whose time series were significantly synchronized to six cycles of stimulation repeated every minute, were observed at striatum in close proximity to the stimulation site (Figs. 4B and C and Fig. S4C, Table 1, $n = 9$ of 12 animals). The hemodynamic response function (HRF) created by averaging the BOLD signal over these six cycles showed that the signal quickly increased, plateaued at a constant level until the end of stimulation, and then rapidly decayed back to baseline (Fig. 4D and Fig. S4D). Illumination with yellow light, which does not activate ChR2, failed to evoke responses in regions modulated during blue light stimulation, and increases in BOLD signal were not observed in a control animal transplanted with ChR2-negative cells (Fig. S5). These control experiments confirm that BOLD signals resulted from the direct stimulation of

engrafted, ChR2-expressing cells. However, negative BOLD signals were sometimes observed surrounding the positive response at the site of stimulation, possibly due to heating-induced frequency shifts (Fig. S6, Table 1). To verify that the local BOLD response faithfully reflected neural activity (Kleinfeld et al., 2011; Maier et al., 2008), we conducted *in vivo* extracellular electrophysiology recordings using a custom-built optrode in striatum (Fig. 4E). Optical stimulation resulted in a robust increase in firing rate (two-tailed Wilcoxon signed-rank test, $n = 8$ of 8 neurons over 34 trials, $P < 0.001$; Figs. 4F and G, Table S1), confirming that optical stimulation of transplanted stem cells can elicit robust neural activity that results in a local BOLD signal.

Importantly, optogenetic stimulation of cells at striatum also resulted in a robust BOLD response in remote regions such as hippocampus, where active voxels exhibited a strong synchronization to the six cycles of stimulation (Figs. 4H and I, Fig. S4E, Table 1). The average HRF of these responses exhibited distinct dynamics compared to those observed in striatum, pointing to the possibility of different neuronal firing patterns (Fig. 4J and Fig. S4F). The hippocampus signal increased at a slower rate and decreased more gradually before returning to baseline. To confirm that this BOLD response faithfully reflected neural activity, we performed single unit extracellular recordings in the hippocampus during optical stimulation of engrafted neurons in striatum (Fig. 4K). Strikingly, stimulation resulted in a significant increase in firing rate (two-tailed Wilcoxon signed-rank test, $n = 7$ of 12 neurons over 10 trials, $P < 0.01$; Figs. 4L and M, Table S1). The response was also more gradual than the striatum's firing pattern, in agreement with expectations based on HRF shapes.

In addition to the fMRI activity in hippocampus, BOLD responses exhibiting a strong synchronization to stimulation were found in downstream areas of the brain including cingulate cortex (Fig. 4B and Figs. S7A–D, Table 1), septal nuclei (Figs. S7G and H, Table 1), and retrosplenial cortex (Fig. 4H). Axonal projections from the graft were observed at both the cingulate cortex and septal nuclei (Fig. 3), and single-unit recordings at the cingulate and retrosplenial cortex confirmed neuronal firings synchronized to optical stimulations (Figs. S7E–F and I–K). Collectively, these results show that ofMRI can detect neuronal activity of downstream targets that are modulated by transplanted stem cell-derived neurons upon their functional integration with host circuitry.

4. Discussion

We combined fMRI with *in vivo* optogenetic stimulation of human iPSC-derived neurons to provide the first direct assessment of a neural graft's global functional integration with the central nervous system of a host organism. While previous studies have focused on anatomical or behavioral outcomes, the technology we have developed directly measures the ability of engrafted cells to modulate downstream circuits, and allows these functional connections to be visualized across the whole brain in an intact organism. Importantly, the observed functional activities are largely in agreement with underlying graft-host connections. For example, the response in hippocampus may have been mono- or polysynaptically driven, since axonal projections were observed in both the septal nuclei – a major input structure to hippocampus that was modulated by stimulation and received projections from engrafted cells – as well as in the corpus collosum directly above CA1 (Fig.

3, panels 7 and 8). The identification of axonal projections in cingulate cortex (Fig. 3, panel 4) also supports the BOLD activity observed there. These connections suggest that detected BOLD signals reflect direct neuronal communication between the engrafted population and remote regions.

Another possibility is that remote activity is driven indirectly by endogenous cells in the striatum. While the transplanted cells expressing channelrhodopsin are the only ones that can be directly excited by light, their activation may result in the subsequent release of neurotransmitters in striatum that go on to locally modulate host neurons. However, additional studies in our lab have shown that targeted stimulation of endogenous medium spiny neurons drives dramatically different activations than what was observed here, with strong modulation of cortex, thalamus, and basal ganglia including the subthalamic nucleus and substantia nigra (data not shown). Therefore, given the lack of activity in the canonical basal ganglia circuit, local modulation of host neurons is less likely to be the source of remote activity.

An interesting observation from our electrophysiology experiments was that some engrafted cells which exhibited a very robust response to light stimulation had an otherwise low baseline firing rate (Table S1). This suggests that not all neurons in the engrafted population are tonically active, but instead fire in response to certain stimuli. While the presence of spontaneous action potentials has been conventionally used as a measure of functional integration (Wernig et al., 2008), the technology proposed here uses optogenetic techniques to directly excite engrafted cells. As a result, it allows the functional properties of cells that are not tonically firing to be actively investigated. By offering precise control over the transplanted cells' activity, the proposed technology provides a more general approach for identifying graft-host interactions than previous methods.

The human iPSCs employed in our study exhibited robust integration, as demonstrated by the cells' survival, continued ChR2 expression, widespread axonal projections to various brain structures, and ability to drive local and remote responses. While transplantation with a rodent cell line may have yielded better integration with host circuitry, we chose to transplant human cells to offer greater translational potential and provide groundwork for the phenotyping of human cells *in vivo*. In particular, we sought to preemptively overcome challenges such as successful ChR2 expression in human cells and the possibility of immune rejections upon transplantation. Future studies that use this approach with pathological animal models may also yield a higher level of integration than what was observed here. However, we investigated the graft-host integration process in a disease-free context to avoid biases and provide a general framework that can be used in any disease model.

While there are several potential mechanisms underlying the therapeutic efficacy of neural transplants (Lindvall et al., 2004), the reconstruction of neural circuitry is what makes these therapies so uniquely promising. We found that selective stimulation of transplanted neurons can evoke local and remote changes in the BOLD signal associated with neural activity, thus providing a direct measure of a graft's functional integration with host circuitry *in vivo*. These findings demonstrate that selective optical stimulation of neural grafts *in vivo* combined with robust, high-resolution fMRI readouts can be used to non-invasively measure

the spatiotemporal dynamics of graft-host neural circuitry at both the local transplantation site and at downstream targets. Because fMRI is a non-invasive imaging technique that allows *in vivo* investigation of brain function, our proposed approach provides a major advantage by enabling the global and causal function of transplanted stem cells to be evaluated in an intact, fully assembled brain. The proposed approach can in principle also be directly translated to assess transplantation effects in human brains.

Future studies may utilize this technique for the development and optimization of stem cell based therapies. For example, systematic comparison of ofMRI-visualized activity resulting from different protocols of differentiation or transplantation may help identify how parameters lead to different patterns of circuit activity or reveal potential mechanisms of the graft-host integration process. Comparing the dynamics elucidated with ofMRI and phenotypes measured with behavioral readouts may also provide insight into how the circuits formed by regenerated tissue affect behavioral outcomes. In addition, stimulation of axons expressing ChR2 at remote regions (Lee et al., 2010b) could help elucidate the functional role of specific remote projections formed by the graft. Finally, by combining stimulation of stem cell-derived neurons with that of host neurons, our technique may be adapted to directly compare the network properties of engrafted and endogenous neuronal populations. Together, these ofMRI-enabled studies will provide the much-needed information to advance stem cell based therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

iPSC	induced pluripotent stem cell
hESC	human embryonic stem cell

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Highlights

- Induced pluripotent stem cells (iPSCs) are engineered to express channelrhodopsin
- Stem cells are differentiated to neurons and transplanted in rat brain
- Selective optical stimulation of neural grafts *in vivo* evokes global fMRI signals
- Neural graft's causal function is non-invasively visualized across whole brain

Fig. 1.

Human iPSC-derived neurons stably express ChR2 and are optically excitable in culture. (A) Diagram illustrating the generation of ChR2-expressing neurons from human induced pluripotent stem cells (iPSCs). iPSCs were cultured on matrigel (B), transfected with ChR2-EYFP, FACS purified, and differentiated to neurons. Scale bar, 200 μ m. (C) Robust ChR2 expression was selected for based on high EYFP expression through FACS purification. Numbers in the upper left corner of each panel indicates the percentage of samples above the diagonal line. (D) After 23 days of *in vitro* differentiation through growth factor patterning, iPSC-derived cell cultures co-express ChR2 and the neuron-specific marker β 3-tubulin. Morphologically, the cells have many projections and form networks with neighboring neurons, suggesting that they are progressing to maturation. Scale bar, 100 μ m. (E) Neural stem cells (NSCs) and neurons were manually isolated from culture for transplantation. White arrowheads indicate neural rosettes, self-organizing clusters of neural stem cells. Scale bars, 200 μ m. (F) *In vitro* current clamp recordings show robust, selective action potential excitation of isolated neurons in response to repeated $\sim$ 1 s pulses of continuous photostimulation with 473 nm light. Among the 9 cells that were tagged and recorded, 4 generated action potentials and 5 generated voltage deflections in response to light.

Fig. 2.

Histological characterization of ChR2-expressing, iPSC-derived grafts transplanted to striatum ($n = 4$ rats). (A) Diagram of cell transplantation to the rat striatum (top) and *in vivo* MRI anatomical image of probe tract in a representative animal (bottom). White dashed line indicates the divide between dorsal and ventral striatum (Voorn et al., 2004). Arrow indicates tip of probe tract. (B) Coronal section demonstrates the robust expression of EYFP-ChR2 and large size of the engrafted cell population throughout striatum. White arrowheads indicate axonal bundles entering the corpus collosum (CC). (C) Engrafted cells co-express ChR2 and human-specific nuclear antigen marker (NuMA). (D) Approximately 15% of engrafted cells expressing ChR2 also stain positive for NeuN (indicated by white arrowheads), suggesting they have differentiated into fully mature neurons. (E-G) ChR2-positive axons at the graft-host interface and extending from the graft co-express the human-specific neural cell adhesion molecule (hNCAM), polysialylated-neural cell adhesion molecule (PSA-NCAM), and neuron-specific class III beta-tubulin marker Tuj1. (H) The neural stem cell marker nestin is mostly absent from engrafted populations. Inset shows a single cell co-expressing nestin and ChR2; scale bar, 5 μ m. (I) Ki67-expressing cells are very sparse within the graft, while doublecortin (DCX) is observed throughout.

Fig. 3.

Characterization of axonal projections to close and remote target areas ($n = 3$ rats).

Rectangles on wide-field grayscale images (left column) correspond to zoomed-in confocal panels numbered 1 through 10. Graft axons project along white matter tracts (1, 2, 3, 8, 10) and to specific grey matter zones (4, 5, 6, 7, 9). White arrowheads indicate identified axons. Dashed line in panel 1 indicates interface between striatum and corpus callosum (CC). All panels show EYFP expression. Scale bars for wide-field images, 500 μm . Scale bar for panel 1 zoom-out, 100 μm . All other scale bars, 20 μm .

Fig. 4. Stimulation of transplanted iPSC-derived neurons *in vivo* evokes local and remote fMRI BOLD signals associated with neural activity. (A) Diagram of *in vivo* fMRI experiment and location of provided fMRI slices. (B) Representative activation maps show the striatum BOLD signal (circled) in a single animal resulting from six cycles of optical stimulation. Active voxels are color coded according to the strength of their synchronization with the six cycles of stimulation, as measured by coherence. Asterisk on slice 3 indicates approximate location of stimulation. Number in lower left corner of each map indicates approximate AP position (mm) from bregma. Scale bar, 2 mm. (C and D) Average time series and hemodynamic response function (HRF) of active striatum voxels detected in $n = 7$ of 10 animals. Time series were extracted from active voxels in the images of individual animals. Error bars in C represent one s.e.m across animals. Error bars in D represent one s.e.m. across stimulation cycles for the average six-cycle time series. Blue bars represent 20 s stimulation periods. (E) Diagram of simultaneous *in vivo* recording and optical stimulation in striatum. Inset shows spiking activity of recorded neurons. (F and G) Peristimulus time

histogram and corresponding quantification of firing rate from a representative neuron ($n = 34$ trials, *** $P < 0.001$ pre vs. light, $P = 0.73$ pre vs. post, n.s. not significant, two-tailed Wilcoxon signed-rank test). Neuronal firing rate increased during stimulation for 8 out of 8 neurons recorded (Table S1). Values are mean $\pm$ s.e.m. (H) Representative activation maps, taken from the same dataset as those shown in B, show robust BOLD activity at hippocampus (Hp). Numbers in the lower left corner of each map indicate approximate AP position (mm) from bregma. Scale bar, 2 mm. (I and J) Average time series and HRF of active hippocampus voxels detected in $n = 4$ of 10 animals. Blue bars represent 20 s stimulation periods. (K) Diagram of *in vivo* hippocampus recordings during optical stimulation in striatum. Inset shows spiking activity of recorded neurons. (L and M) Peristimulus time histogram and corresponding quantification of firing rate from a representative neuron ($n = 10$ trials, ** $P < 0.01$ pre vs. light, $P = 0.63$ pre vs. post, n.s. not significant, two-tailed Wilcoxon signed-rank test). Neuronal firing rate increased in hippocampus during optical stimulation at striatum for 7 of 12 recorded neurons. One neuron exhibited a decrease in firing rate, and 4 exhibited no effect (Table S1). Values are mean $\pm$ s.e.m.

Analysis of individual animal results for fMRI experiments with iPSC- and hESC-derived grafts. Columns indicate the average number of active voxels identified in each region and their corresponding amplitude. Amplitude was defined as the difference between the maximum and minimum values of the mean HRF. Negative BOLD signals were sometimes observed at the site of stimulation, possibly due to heating-induced frequency shifts (Christie et al., 2012). This effect could offset the positive BOLD changes driven by neural activity and explain the lack of local activation observed in some animals. Sessions lacking such activity were excluded from the generation of time series.

Table 1

Cell Line/Animal ID	Active striatum voxels (amplitude)	Active hippocampus voxels (amplitude)	Active cingulate cortex voxels (amplitude)	Active septal nuclei voxels (amplitude)	Local negative voxels	Included sessions
iPSC -1	10 (1.70%)	33 (1.24%)	28 (0.96%)	11 (1.33%)	8	7 of 10
iPSC -2	0	0	0	16 (1.57%)	0	1 of 4
iPSC -3	31 (1.51%)	8 (1.58%)	0	0	35	4 of 4
iPSC -4	3 (1.84%)	13 (1.56%)	16 (0.89%)	0	0	2 of 5
iPSC -5	16 (1.96%)	4 (1.80%)	93 (0.70%)	18 (0.86%)	2	3 of 4
iPSC -6	0	0	25 (0.84%)	0	37	2 of 5
iPSC -7	0	0	21 (0.63%)	8 (1.34%)	11	3 of 3
iPSC -8	24 (2.39%)	0	0	0	0	1 of 1
iPSC -9	38 (3.92%)	0	0	0	0	1 of 4
iPSC -10	17 (2.87%)	0	0	0	0	4 of 4
hESC -1	17 (2.4%)	36 (2.04%)	60 (0.97%)	13 (1.33%)	12	7 of 10
hESC -2	4 (1.31%)	0	7 (1.39%)	0	37	2 of 6