Immature electrophysiological properties of human-induced pluripotent stem cell-derived neurons transplanted into the mouse cortex for 7 weeks

Tasuku Kayama[a], Kazuki Okamoto[a], Mengxuan Gao[a], Yuji Ikegaya[a,b] and Takuya Sasaki[a,c]

The transplantation of human-induced pluripotent stem cell (hiPSC)-derived cells has emerged as a potential clinical approach for the treatment of brain diseases. Recent studies with animal disease models have shown that hiPSC-derived neurons transplanted into the brain, especially the nigrostriatal area, could restore degenerated brain functions. Further works are required to test whether hiPSC-derived neurons can also gain functional properties for other cortical areas. In this study, hiPSC-derived neurospheres were transplanted into the adult mouse hippocampus and sensory cortex. Most transplanted hiPSC-derived neurons expressed both Nestin and NeuN at 7 weeks after transplantation. Whole-cell patch-clamp recordings from brain slices indicated that transplanted cells showed no action potentials upon current injection and few small inward currents, indicating that hiPSC-derived neurons did not become functionally mature within these time periods. *NeuroReport* **00:000–000**

NeuroReport 2018, **00**:000–000

Keywords: action potential, cortex, hiPSC neurons, transplantation

[a]Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, [b]Center for Information and Neural Networks, Suita City and [c]Precursory Research for Embryonic Science and Technology), Japan Science and Technology Agency, Kawaguchi, Japan

Correspondence to Takuya Sasaki, PhD, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo 113-0033, Japan
Tel: +81 358 414 783; fax: +81 358 414 786;
e-mail: tsasaki@mol.f.u-tokyo.ac.jp

Received 23 October 2018 **accepted** 19 November 2018

Introduction

The transplantation of human-induced pluripotent stem cell (hiPSC)-derived neurons in the brain is expected to be a clinical application for neurodegenerative diseases. Recent studies with animal models of Parkinson's disease have shown that induced pluripotent stem cell-derived neurons transplanted into the nigrostriatal area differentiate into functional dopamine neurons and ameliorate Parkinson-like behavioral deficits [1–4].

Similarly, the transplantation of induced pluripotent stem cell-derived neurons into cortical areas is potentially a promising therapy for the replacement of damaged neural circuits in neurodegenerative disorders such as Alzheimer disease [5], traumatic brain injury, and brain ischemia [6]. Several pioneering studies have recently shown that hiPSC-derived cortical neurons transplanted into the lesioned adult mouse cortex expressed mature neuron markers such as NeuN [7] and can generate action potentials and/or receive synaptic transmission [8], suggesting that the transplanted hiPSC-derived cortical neurons become functionally mature. These studies offer a crucial milestone for the potential use of transplantation of hiPSC-derived cortical neurons for the reassembly of the cortex.

The electrophysiological studies were carried out up to 6 months after the transplantation [8]. It remains unclear whether transplanted neurons can gain mature functional properties and connect with the host cortical network within shorter time periods, such as a few weeks and months. At least under culture conditions *in vitro*, it has been shown that hiPSC-derived neurons can acquire the ability to generate clear action potentials [9,10]. To understand the detailed time courses of cell differentiation following transplantation in in-vivo living animals, we tested whether time periods up to 7 weeks are sufficient for hiPSC-derived neurons transplanted into the cortex to gain functionally mature characteristics. The results will be a step for regenerative research with animal models and the preclinical development of cell therapy strategies.

Materials and methods

Animal ethics

All animal experiments were conducted with the approval of the animal experimental ethics committee at the University of Tokyo (approval number: P29-9; P29-13) and in accordance with the NIH guidelines for the care and use of animals. Nonobese diabetic/severe combined immunodeficiency mice were purchased from the Jackson Laboratory (JAX stock number: 001303, Bar Harbor, Maine, USA). The animals were 4–8 weeks old, weighed 10–25 g, and maintained with free access to water and food under inverted 12-h light/12-h dark conditions (light from 8 pm to 8 am).

0959-4965 DOI: 10.1097/WNR.0000000000001178

Culture of hiPSC-derived neurons

Cryopreserved hiPSC-derived neurons (RCESDN002; Reprocell, Yokohama, Japan) were thawed with thawing medium (RCESDN305; Reprocell). The cell suspensions were centrifuged at 400*g* for 5 min and the supernatant was removed. Then, hiPSC-derived neurons were dissolved in maturation medium (RCESDN302; Reprocell) and plated on poly-D-lysine-coated 12 mm glass coverslips (Neuvitro Corp., Vancouver, Washington, USA) and Aggrewell (cat#34815; Reprocell) for dissociated culture and neurosphere methods, respectively, at a density of $1–3 \times 10^5$ cells/well with maturation medium. For virus infection, a 3-μl virus solution (rAAV5/CamkII-hchR2 (H134R)-EYFP-WPRE-PA; UNC GTC Vector Core, Chapel Hill, North Carolina, USA) was added to the cultures when plating the neurosphere them so that the final concentration of the virus was $1–2 \times 10^{10}$ Vg/ml. Half of the culture medium was replaced with a new maturation medium twice weekly.

For transplanting hiPSC-derived neurons into the mouse brain, we obtained a 1-ml neurosphere solution containing about $1–3 \times 10^5$ cells from one well using cell strainers (cat#27215; Stem Cell Technologies, Vancouver, British Columbia, Canada) after 1–3 weeks of cultivation. The neurosphere solution was centrifuged at 100*g* for 1 min and the supernatant was removed. The neurospheres were dissolved in a 50-μl maturation medium.

For immunohistochemistry, the cultured hiPSC-derived neurons on a glass dish were fixed with 4% paraformaldehyde at 37°C for 30 min.

Transplantation of hiPSC-derived neurons

Mice were anesthetized with pentobarbital (20 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally) and fixed in a stereotactic frame (Narishige Co., Ltd, Tokyo, Japan). The body temperature was maintained using a heating pad. A midline incision was made above the skull, and circular craniotomies ~0.7 mm in diameter were made with a high-speed drill (SD-102; Narishige Co., Ltd) at coordinates of 1.9 mm posterior and 1.5 mm bilateral to the bregma for the hippocampus. A 5-μl Hamilton syringe (tip inner diameter = 310 μm; Hamilton Company, Reno, Nevada, USA) was loaded with the maturation medium solution containing ~1×10^5 hiPSC-derived neurons and the tip of the syringe was then lowered into the hippocampus through the craniotomy at a depth of 1.6 mm. A total of 2 μl solution (~4000 cells) was injected manually into the hippocampus at a rate of 500 nl/min. To prevent backflow of the injected cells, the syringe was held in place in the tissue for 5 min after the injection. After the syringe was withdrawn, the incision was sutured. Following surgery, each mouse was housed individually in transparent Plexiglass with free access to water and food.

Immunostaining

The mice were perfused intracardially with cold 4% paraformaldehyde in 25 mM PBS and decapitated. The brains were coronally sectioned at a thickness of 200 μm.

For cultures and brain slices, the fixed samples were rinsed with PBS and then permeabilized in 100 mM PBS with 0.1 and 0.3% Triton X-100 and 5 and 10% goat serum (Vector Laboratories, Burlingame, California, USA) at room temperature for 60 min, respectively. They were then incubated with primary mouse anti-Human nuclei (1 : 200 for brain slice; 1 : 1000 for cell culture; Merck Millipore, Burlington, Massachusetts, USA), rabbit anti-NeuN (1 : 500 for brain slice and 1 : 300 for cell culture; Abcam, Cambridge, UK), rabbit anti-MAP2 (1 : 200 for brain slice and 1 : 1000 for cell culture; Abcam), chicken anti-GFP (1 : 1000; Abcam), mouse anti-Nestin (1 : 200; Abcam), rat anti-Nestin (1 : 300 for brain slice and 1 : 200 for cell culture; Abcam), and guinea-pig anti-vGlut1 (1 : 500; Synaptic Systems, Goettingen, Germany), in 100 mM PBS, 0.1% and 0.3% Triton X-100, and 5 and 10% goat serum for one overnight period at 4°C, respectively. After they were rinsed with PBS, they were then labeled with secondary anti-Mouse IgG Alexa-405, Alexa-488, and Alexa-594 (1 : 500; Thermo Fisher Scientific, Waltham, Massachusetts, USA), secondary anti-Rabbit IgG Alexa-405, 488, and 647 (1 : 500; Thermo Fisher Scientific), secondary anti-Chicken IgG Alexa-488 (1 : 500; Thermo Fisher Scientific), secondary anti-Guinea pig IgG Alexa-488 (1 : 500; Thermo Fisher Scientific), and secondary anti-Rat IgG Alexa-647 (1 : 500; Thermo Fisher Scientific) in 100 mM PBS for 6 h and one overnight for dissociated cultures and brain slices, respectively. For cultures and brain slices, the samples were mounted with vectashield containing DAPI (Funakoshi Co., Ltd, Tokyo, Japan) and embedded in Permafluor (Thermo Fisher Scientific), respectively. Images were acquired at a *Z*-depth interval of 2 μm using a confocal laser-scanning microscope (BX51-FL; Olympus, Tokyo, Japan) with a water-immersion objective lens (×10, 0.4 NA; ×20, 0.75 NA; ×40, 0.95 NA).

Electrophysiology

Mice were anesthetized with isoflurane and then decapitated. The brains were removed and placed in an ice-cold oxygenated solution consisting of (in mM) 222.1 sucrose, 27 $NaHCO_3$, 1.4 NaH_2PO_4, 2.5 KCl, 1 $CaCl_2$, 7 $MgSO_4$, and 0.5 ascorbic acid. The brains were sliced at a thickness of 400 μm coronally using a VT1200S vibratome (Leica, Wetzlar, Germany). Slices were allowed to recover at room temperature for at least 30 min while submerged in a chamber filled with an oxygenated solution consisting of (in mM) 127 NaCl, 26 $NaHCO_3$, 1.6 KCl, 1.24 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, and 10 glucose. All recordings were performed at room temperature. Borosilicate glass pipettes (3 – 6 MΩ) were filled with a solution containing (in mM) 135 K-gluconate, 4 KCl, 0.3 EGTA, 10 HEPES, 10 Na_2-phosphocreatine, 4 MgATP, and 0.3 Na_2GTP with 2.0

biocytin. The signals were gained five-fold, low-pass filtered at 1 kHz, and digitized at 20 kHz. To label the cell morphology of recorded cells, the slice sections were incubated with 2 μg/ml streptavidin–Alexa Fluor 594 conjugate and 0.2% Triton X-100 for 6 h, followed by incubation with 0.4% NeuroTrace 435/455 Blue Fluorescent Nissl Stain (N21479; Thermo Fisher Scientific) overnight.

Statistical analysis

All data are presented as the mean ± SEM.

Results

Expression of neuron markers hiPSC-derived neurons in culture

HiPSC-derived neurons were dissociated and cultivated on a poly-D-lysine-coated glass (Fig. 1a). Immunostaining showed that 87.2 ± 2.9 and 64.3 ± 9.2% of cultured hiPSC-derived neurons at 7 and 14 days *in vitro* (DIV) expressed Nestin, an immature neuron marker, respectively (Fig. 1b). In addition, 49.6 ± 15.6 and 49.6 ± 2.1% of the same sets of hiPSC-derived neurons expressed NeuN, a mature neuron marker, with the co-expression of Nestin and NeuN in 47.7 ± 15.3 and 44.8 ± 2.9% of these hiPSC-derived neurons at 7 or 14 DIV, respectively. In different sets of cultures, 37.9 ± 4.3 and 70.1 ± 5.7% of hiPSC-derived neurons expressed microtubule-associated protein 2 (MAP2) at 7 and 14 DIV, respectively (Fig. 1c).

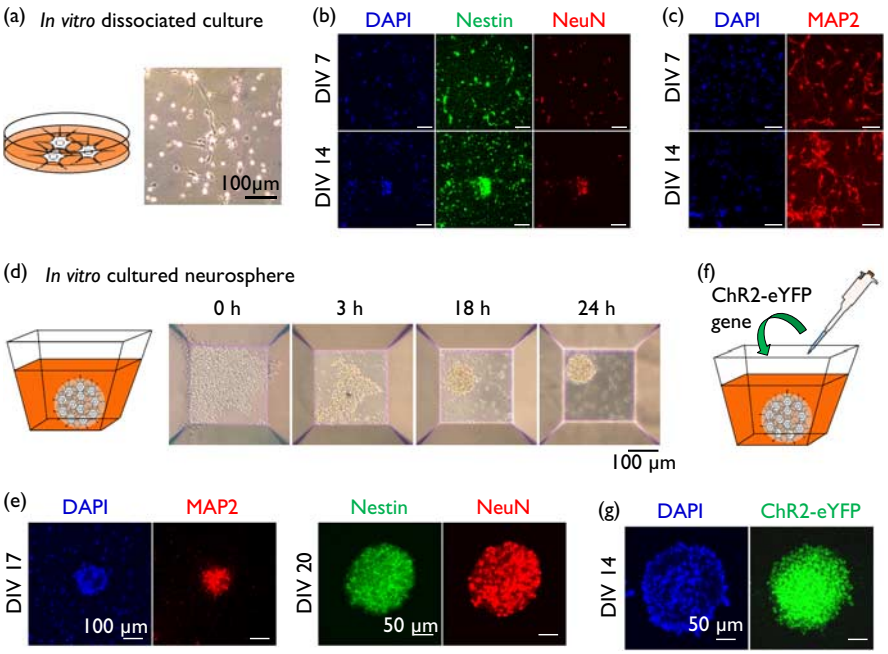
To transplant cultured hiPSC-derived neurons into the mouse cortex, we utilized a neurosphere method in which hiPSC-derived neurons floated on a well plate for several hours so that they spontaneously formed sphere-like cell clusters with diameters of ~ 100 μm consisting of 300–1000 hiPSC-derived neurons (Fig. 1d). These hiPSC-derived neurospheres expressed Nestin, NeuN, and MAP2 (Fig. 1e).

To detect hiPSC-derived neurons in living samples after transplantation into the brain, hiPSC-derived neurons were labeled with a fusion protein of channelrhodopsin 2 (ChR2) and eYFP (ChR2-eYFP) by adding virus solution containing rAAV5/CaMKII-ChR2-eYFP to the culture (Fig. 1f). HiPSC-derived neurospheres showed the clear expression of eYFP fluorescence (Fig. 1g), confirming the successful introduction of marker proteins into cultured hiPSC-derived neurons.

HiPSC-derived neurons transplanted into the cortex express neuron markers

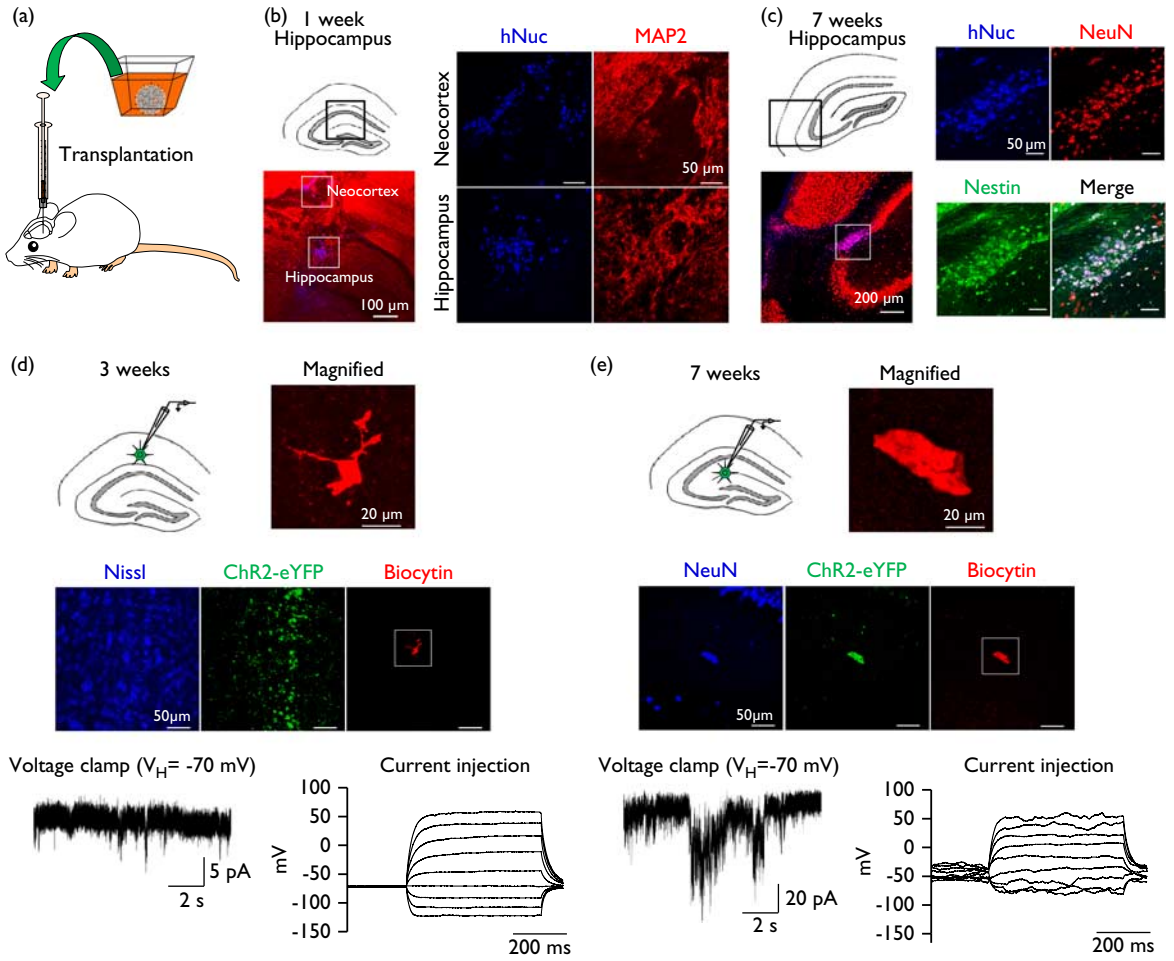
HiPSC-derived neurospheres were transplanted into the mouse cortex (Fig. 2a). For this experiment, transgenic mice with immunosuppression were needed to prevent

Fig. 1



Expression of neuron markers by human-induced pluripotent stem cell (hiPSC)-neurons in cultures. (a, left) A schematic image of hiPSC-derived neurons plated on a poly-D-lysine-coated glass dish. (a, right) A representative bright-field image of hiPSC-derived neurons at 11 days *in vitro* (DIV) in a dissociated culture. Scale bars, 100 μm. (b, c) Representative images of hiPSC-derived neurons immunostained with DAPI, Nestin, NeuN, and MAP2 at 7 and 14 DIV. Scale bars, 100 μm. (d, left) A schematic image of a hiPSC-derived neurosphere. (d, right) Time-lapse imaging of the formation of a neurosphere for 24 h in a culture dish. (e) Representative images of a hiPSC-derived neurosphere immunostained with DAPI, MAP2, Nestin, and NeuN. (f) HiPSC-derived neurospheres were transfected with virus by adding the virus solution containing AAV5-CaMKII-ChR2-eYFP to the cultures. (g) DAPI immunofluorescence and ChR2-eYFP fluorescence.

Fig. 2



Transplanted human-induced pluripotent stem cell (hiPSC)-derived neurons into the mouse cortex were not capable of generating action potentials. (a) A schematic image of transplantation of hiPSC-derived neurospheres into the mouse cortex. (b, left) A transplantation site is indicated by the box in the top panel. The bottom picture shows the indicated area. The neocortical and hippocampal regions indicated by the two white boxes are further magnified in the right panels. (b, right pictures) Images of transplanted hiPSC-derived neurons immunostained with human-specific nuclei (hNuc; blue) and MAP2 (red) at 1 week after transplantation. (c) Same as 'c', but for Nestin (green) at 7 weeks after the transplantation. (d, top) A recording site and a magnified image of a biocytin-injected hiPSC-derived neuron. (d, middle) Images of slices with Nissl or NeuN fluorescence, ChR2-eYFP signals, and a biocytin-injected hiPSC-derived neuron. The area indicated by the white box corresponds with the image in the top panel. (d, bottom left) A representative current trace from an eYFP-expressing hiPSC-derived neuron recorded at −70 mV. (d, bottom right) Membrane potential traces in response to step current injection. No action potentials were generated. (e) Same as 'd', but for 7 weeks after the transplantation.

immunoreaction against transplanted cells. Of 30 mice that were subjected to surgery, 17 mice died within 2 weeks after transplantation. Although the target area of our transplantation was specific to the hippocampus with the tip of the injection syringe at a depth of 1.6 mm, the cell bodies of transplanted cells in the tissue were typically found along the injection track not only in the hippocampus but also the somatosensory cortex above the hippocampus. At 1 or 2 weeks after transplantation, transplanted cells in the cortex expressed MAP2 (Fig. 2b). In addition, 86.9 ± 9.3% of hiPSC-derived neurons expressed NeuN with the co-expression of Nestin and NeuN in 68.1 ± 16.0% of these hiPSC-derived neurons 7 weeks after the transplantation (Fig. 2c).

Transplanted hiPSC-derived neurons did not generate functional electrical activity at least 7 weeks after transplantation

To directly examine whether transplanted cells differentiated to generate functional activity, we labeled hiPSC-derived neurons with ChR2-eYFP as in Fig. 1g and implanted these cells into the brain. Expression of eYFP fluorescence in hiPSC-derived neurons remained stable in the tissue for up to 7 weeks after the transplantation (Fig. 2d and e). After the transplantation, acute cortical slices were prepared including the transplanted area and targeted whole-cell patch-clamp recordings were performed from eYFP-labeled hiPSC-derived neurons. Overall, all two cells from two animals tested at 3 weeks

(Fig. 2d) and all four cells from three animals tested at 7 weeks (Fig. 2e) after transplantation expressed NeuN, but emitted no action potentials upon the injection of rectangular currents (from -60 to $+110$ pA, 500 ms). Voltage-clamp recordings confirmed that these cells had small spontaneous inward currents with a frequency of 0.1 ± 0.05 Hz and an amplitude of 7.2 ± 3.0 pA ($n = 145$ events from four cells at 7 weeks) that appeared to be different from general synaptic currents observed from matured cortical pyramidal cells. These results suggest that at least 7 weeks were insufficient for our transplanted cells to gain functional properties.

Discussion

Our immunofluorescence staining confirmed that most cultured neurospheres and transplanted hiPSC-derived neurons were positive for both Nestin and NeuN, which are widely used immature and mature neuron markers, respectively. This observation raised the possibility that the maturation stage of these transplanted neurons might be intermediate between mature and immature stages and capable of emitting active membrane currents. Contrary to this expectation, no action potentials were detected from these cells, implying that they had not functionally matured. Although a number of studies have evaluated the maturation levels of transplanted hiPSC-derived neurons in the brain using histological markers [11,12], our results suggest that the differentiation of transplanted cells might be evaluated more accurately when histological data are combined with electrophysiological assessments.

Compared with dopaminergic neurons in the midbrain area, the replacement of neurons by hiPSC technology seems to be particularly challenging for cortical areas. Our results showed that 7 weeks were not sufficient for integration of transplanted hiPSC-derived neurons into the naive cortical circuit, which suggests that longer time periods are required to obtain behavioral phenotypes for potential clinical consideration. We need to note that different maturation processes may be observed in cortical areas other than the hippocampus and the somatosensory cortex, which could not be tested in this study. Future research is required to obtain consistent protocols for neuron transplantation into the cortex so that neurons can function in a physiologically relevant way to improve cognitive ability and increase the capacity for neuronal processing in the cortex. Transplantation of hiPSC-derived neurons with hiPSC-derived glial cells and blood vessels or molecular cues such as morphogen and growth factors may be effective to induce faster differentiation and maturation of transplanted cells [13].

Acknowledgements

This work was supported by Kaken-hi (17H05939 and 17H05551), a Grant in Aid for Precursory Research for Embryonic Science and Technology from the Japan Science and Technology Agency.

Conflicts of interest

There are no conflicts of interest.

References

1 Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**:861–872.
2 Kikuchi T, Morizane A, Doi D, Magotani H, Onoe H, Hayashi T, *et al.* Human iPS cell-derived dopaminergic neurons function in a primate Parkinson's disease model. *Nature* 2017; **548**:592–596.
3 Wernig M, Zhao JP, Pruszak J, Hedlund E, Fu D, Soldner F, *et al.* Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. *Proc Natl Acad Sci USA* 2008; **105**:5856–5861.
4 Hallett PJ, Deleidi M, Astradsson A, Smith GA, Cooper O, Osborn TM, *et al.* Successful function of autologous iPSC-derived dopamine neurons following transplantation in a non-human primate model of Parkinson's disease. *Cell Stem Cell* 2015; **16**:269–274.
5 Fujiwara N, Shimizu J, Takai K, Arimitsu N, Ueda Y, Wakisaka S, *et al.* Cellular and molecular mechanisms of the restoration of human APP transgenic mouse cognitive dysfunction after transplant of human iPS cell-derived neural cells. *Exp Neurol* 2015; **271**:423–431.
6 Hermanto Y, Sunohara T, Faried A, Takagi Y, Takahashi J, Maki T, *et al.* Transplantation of feeder-free human induced pluripotent stem cell-derived cortical neuron progenitors in adult male Wistar rats with focal brain ischemia. *J Neurosci Res* 2018; **96**:863–874.
7 Mohamad O, Drury-Stewart D, Song M, Faulkner B, Chen D, Yu SP, *et al.* Vector-free and transgene-free human iPS cells differentiate into functional neurons and enhance functional recovery after ischemic stroke in mice. *PLoS One* 2013; **8**:e64160.
8 Avaliani N, Sorensen AT, Ledri M, Bengzon J, Koch P, Brustle O, *et al.* Optogenetics reveal delayed afferent synaptogenesis on grafted human-induced pluripotent stem cell-derived neural progenitors. *Stem Cells* 2014; **32**:3088–3098.
9 Kayama T, Suzuki I, Odawara A, Sasaki T, Ikegaya Y. Temporally coordinated spiking activity of human induced pluripotent stem cell-derived neurons co-cultured with astrocytes. *Biochem Biophys Res Commun* 2018; **495**:1028–1033.
10 Odawara A, Katoh H, Matsuda N, Suzuki I. Physiological maturation and drug responses of human induced pluripotent stem cell-derived cortical neuronal networks in long-term culture. *Sci Rep* 2016; **6**:26181.
11 Hargus G, Cooper O, Deleidi M, Levy A, Lee K, Marlow E, *et al.* Differentiated Parkinson patient-derived induced pluripotent stem cells grow in the adult rodent brain and reduce motor asymmetry in Parkinsonian rats. *Proc Natl Acad Sci USA* 2010; **107**:15921–15926.
12 Doi D, Samata B, Katsukawa M, Kikuchi T, Morizane A, Ono Y, *et al.* Isolation of human induced pluripotent stem cell-derived dopaminergic progenitors by cell sorting for successful transplantation. *Stem Cell Reports* 2014; **2**:337–350.
13 Mansour AA, Goncalves JT, Bloyd CW, Li H, Fernandes S, Quang D, *et al.* An in vivo model of functional and vascularized human brain organoids. *Nat Biotechnol* 2018; **36**:432–441.