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Human Neural Progenitor Cells Incorporate into Functional Network in Mouse Hippocampus

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Abstract

Human neural progenitor cell (NPC) transplantation into mouse brain has been widely used as an in vivo human-relevant model system. The integration of differentiated human cells or tissue with local circuit has also been intensively investigated. However, as part of the central nervous system which governs the behaviors of living organisms, evidence on whether xenografted neurons are responsive to mouse behavior in intended ways is still poor. In this work, we transplanted iPSC-derived NPCs (iNPCs) into the hilus region of mouse hippocampus and monitored their survival, migration, differentiation and integration in different host mouse lines for different timespan. We found that iNPCs survived for up to two months in C57BL6 mice and for more than six months in Rag2 KO immunodeficient mice. To assess functional integration, we performed contextual fear conditioning with Rag2 KO xenograft mice and examined endogenous Fos protein expression. Dentate gyrus neurons are largely silent and sparsely activated during contextual information learning. We found that fear conditioning training induces Fos gene expression in iNPC-differentiated neurons 6 months after transplantation. The result suggests that behavior-responsive functional integration of human xenografted neurons in mouse hippocampus can be formed after long-time integration.

Keywords

iNPCs; hippocampus xenotransplantation; functional integration.

1. INTRODUCTION

Human NPC transplantation into mouse brain has increasingly been used to model neurological diseases [1-8]. It can recapitulate human-specific pathological progression while still stay in a living organism, not a petri dish, and in connection with host immune, metabolism and homeostasis system [2, 4]. The integration of transplanted cells or organoids with local neuronal network has been intensively examined using imaging and electrophysiology. However, their response to behavior, the core function of brain, remains insufficiently understood. Besides disease modeling, cell transplantation also holds great promise in cell therapy—supplement of healthy cells to rescue diseased ones in different organs/tissues [3, 9]. This is particularly important in the central nervous system, where neurons are terminally differentiated and have limited regenerative capacity.

Entorhinal-hippocampal circuit is one of most reliable behavior-induced neuronal network in the brain [10]. Hippocampal dentate gyrus (DG) neurons which receive projections from entorhinal cortex through perforant pathway are activated immediately after mice are put into a novel environment [11]. Activated DG neurons express immediate early genes (IEGs), such as Fos, and can be specifically labeled for later manipulation using Fos promoter-driven chemo- or

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optogenetic tools [12-16]. Neurotoxin depletion of Fos-expressing neurons abolishes mouse's capability to remember contextual information after learning [15]. In contrast, optogenetic activatio of these population of neurons can retrieve a certain episodic memory [13], or even create a false one [12].

In this study, we transplanted iNPCs into the hilus region of mouse hippocampus and monitored their survival, migration, differentiation and integration into local network. We found that six months after transplantation, iNPCs migrated, differentiated, and incorporated into dentate gyrus neurons. Notably, these iNPC-derived neurons responded to contextual learning by expressing Fos following fear conditioning training. The results suggest that xenografted human neurons can functionally integrate into the hippocampal network.

2. METERIALS AND METHODS

2.1. Subjects

C57BL6 mice were purchased from Charles River Laboratories International Inc., Beijing, China. Rag2 KO mice were purchased from Cyagen Biosciences (Suzhou) Inc., China. All mice were housed at Oujiang Laboratory animal facility following the guidelines of institutional animal care and use committee (IACUC). iNPCs used in this study were purchased from Axol Bioscience (catalog number ax0015). To obtain EGFP-expressing iNPCs, the cells were transduced with rLV-CAG-EGFP (purchased from Wuhan Shumi Brain Science and Technology Co., Ltd., Wuhan, China; viral titer >2e+8 TU/ml), and stable EGFP expression was confirmed. The transduced iNPCs were stereotactically injected into the dentate gyrus (DG) of the mouse hippocampus.

2.2. Antibodies

The following primary antibodies were used for immunofluorescence staining: Rabbit polyclonal anti-Fos antibody (Abcam, AB190289; 1:400), Rabbit monoclonal anti-NeuN antibody (HUABIO, ET1602-12; 1:1000), Rabbit polyclonal anti-MAP2 antibody (Proteintech, 17490-1-AP; 1:200), Mouse monoclonal anti-human nuclear antigen antibody (Abcam, ab191181; 1:400), and Mouse monoclonal anti-PSD-95 antibody (Santa Cruz Biotechnology, sc-71935; 1:100). AlexaFlour 488 (Invitrogen; 1:500), AlexFlour568 (Invitrogen;1:500).

2.3. Mouse stereotactic surgery

C57BL6 mice stereotactic surgery was done in regular lab environment. Rag2 KO mice stereotactic surgery was done in specific-pathogen free mouse facility. Before surgery, mice were anesthetized using isoflurane in an enclosed chamber. During surgery, mice were kept anesthetized with isoflurane mask on. A 1 cm longitudinal incision was cut in the middle of mouse sculp to expose the bregma and lambda points of the skull. Bregma point, lambda point, left and right parietal bones were adjusted on a horizontal plane. A 0.5 mm2 hole was drilled at coordinates (AP = −2.06 mm, ML = −1.2 mm) when the bregma point was set as (AP = 0, ML = 0). Indicated amount of hNPCs in PBS were injected through a glass capillary attached on Nanoject III microinjector (Drummond Scientific Co.) at the depth of 2 mm (DV = -2 mm) with a speed of 2 nl/s. The capillary was withdrawn slowly 5 minutes after injection finished. The incision on the sculp was sutured with absorbable surgical thread. After surgery mice were given dexamethasone and put back to their home cage.

2.4. Mouse perfusion and immunofluorescence staining

Mice at different time points after hNPCs injection were deeply anesthetized using high-concentration isoflurane for transcardiac perfusion. Ice-cold PBS was used to clear the blood and ice-cold 4% polyformaldehyde (PFA) was used for initial fixation of brain tissue before dissection. Dissected mouse brain was emerged in 4% PFA and kept at 4 °C overnight for

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thorough fixation. Fixed brains were dehydrated in 30% sucrose also at 4 °C for two days. Dehydrated brains were embedded in O.C.T. Compound from Sakura Finetek and frozen to -20 °C inside cryostat chamber before section into 20 μm thickness coronal slices. Brain slices were dry-attached on glass slides for microscope observation or immunofluorescence staining. Immunofluorescence staining was done with blocking (1% BSA, 10% goat serum and 0.25% Triton X-100 in PBS) at room temperature for 1 hour, followed by primary antibody incubation at 4 °C overnight and secondary antibody incubation (1:500 in PBS plus 1% goat serum) at room temperature for 1 hour. Immuno-stained samples were mounted with DAPI-containing medium (Beyotime biotechnology China, P0131) before coverslips enclosing. Microscope images were taken using Olympus Fluoview FV3000 confocal microscope.

2.5. Novel environment exploration

Mice were taken out from home cage and put in an 8-arm maze for exploration. The maze is new to experimental mice and its top is open to allow mice see distant visual cues. One hour later, mice were taken out and anesthetized for perfusion and immunofluorescence staining.

2.6. Contextual fear conditioning

Mice were taken out of home cage and put in a fear conditioning chamber for a total of 500 s. During the session, a tone stimulus (20 s, 75 dB, 2,000 Hz) was presented at 180 s, 260 s, 340 s, and 420 s. Each tone co-terminated with a foot shock (2 s, 0.75 mA). After training, mice were put back into home cage after fear conditioning training for another 1 hour to allow immediate early gene expression. Mice finished fear conditioning experiment were anesthetized and perfused for immunofluorescence staining.

3. RESULTS

3.1. Survival and migration of transplanted iNPCs in mouse hippocampus

To monitor the fate of transplanted iNPCs in the mouse hippocampus, we used iNPCs with stable EGFP expression (Figure 1A). A total of 500,000 purified iNPCs were stereotactically injected into the hilus region of mouse hippocampus (Figure 1A). Wild-type (WT) mice were initially used to evaluate transplantation efficiency and early cell survival. One day after injection, EGFP-positive iNPCs were detected in the hilus region, indicating successful engraftment. Transplanted cells were observed at 1 day, 3 days, 1 week, and 1 month post-injection (Figure 1C-D and Figure 3C-D). However, 2.5 months after injection, no iNPS was detected in the transplantation site, suggesting an immune system-mediated clearance (Figure 1C).

We instead used Rag2 KO immunodeficient mice for long-term analysis. After iNPCs transplantation, mice were maintained in a pathogen-free facility. Transplanted iNPCs can survive up to 6 months in Rag2 KO hippocampus (Figure 1C, Figure 2 and Figure 3C-D). We examined iNPS migration in transplanted mouse hippocampus and found that iNPCs started to migrate into the granular zone of dentate gyrus (DG) 3 days after injection (Figure 1D). Two months after injection, iNPCs exhibited neurite-like projections and migrated further into the granular cell layer, suggesting an early neuronal differentiation within host hippocampus (Figure 1E).

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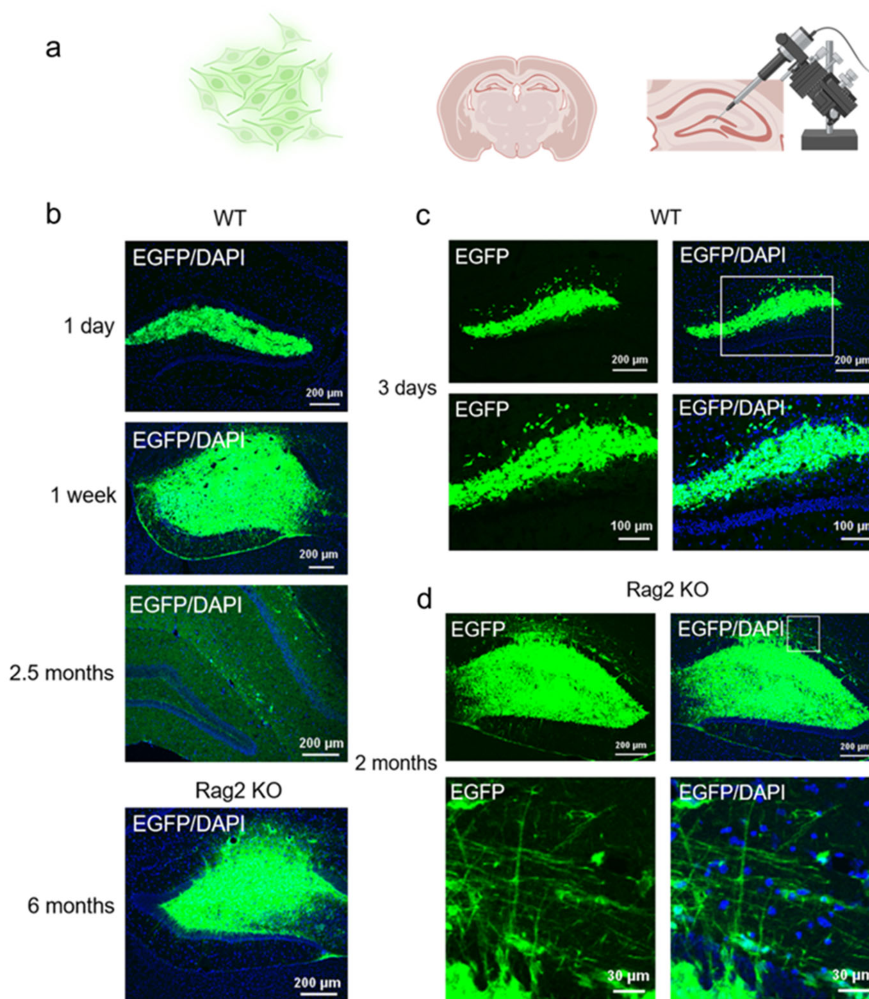

Figure 1. Transplantation and survival of EGFP-expressing iPSC-derived NPCs in mouse hippocampus. (a) Schematic illustration of EGFP+ iPSC-derived neural progenitor cells (iNPCs) and their stereotaxic injection into the dentate gyrus (DG) of the mouse hippocampus. (b) Survival of EGFP-positive iNPCs in the hilus region of the hippocampus at different time points in wild-type (WT) and Rag2 KO mice (green: EGFP; blue: DAPI).(c) Growth and morphology of iNPCs in WT mouse hippocampus at 3 days post-injection. Lower panels show higher-magnification images of the indicated regions (green: EGFP; blue: DAPI).(d) Growth and morphology of iNPCs in Rag2 KO mouse hippocampus at 2 months post-injection. Lower panels show higher-magnification images of the indicated regions (green: EGFP; blue: DAPI).

3.2. Differentiation and synaptic integration of transplanted iNPCs in Rag2 KO mice

To confirm the differentiation state of transplanted iNPCs, we performed immunofluorescence staining using neuronal markers in Rag2 KO mice 6 months after transplantation (Figure 2A-B). At low magnification level, we observed EGFP-positive iNPCs were largely overlapped with NeuN-positive cells, indicating their integration into the neuronal layer (Figure 2A). At higher magnification, we noticed that a subset of human cells as indicated by human nuclear antigen (hNA) immunofluorescence staining colocalize with MAP2 expressing neurons displaying typical neuronal morphology with extended dendritic structures (Figure 2B). We confirmed that these human neuron population is EGFP positive, indicating their iNPC origin (Figure 2C).

DG granular cells receives axonal projection from entorhinal cortex through synapses formed between their dendritic spines and entorhinal neuron axonal boutton. To assess potential synaptic integration of iNPCs, we first examined their dendritic projections and found their

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extension in DG molecular layer (Figure 2D). We then did postsynaptic marker PSD-95 immunofluorescence staining to examine dendritic spine formation in iNPC-derived neurons. We confirmed that PSD-95 is mostly detected in the molecular layer and distributes in punctate pattern (Figure 2D). Higher-magnification imaging revealed overlap between EGFP-positive processes and PSD-95 puncta, suggesting that mature synapses have formed between transplanted iNPC-derived neurons and entorhinal neurons (Figure 2D, lower panels).

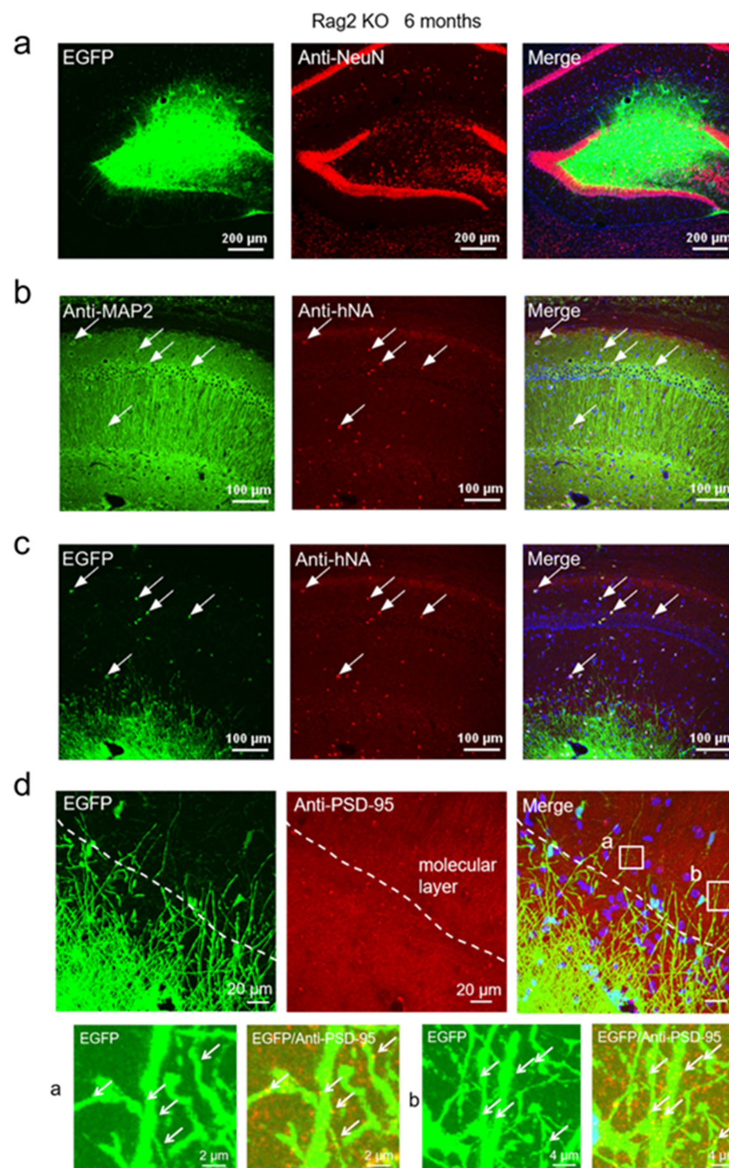

Figure 2. Differentiation and synaptic integration of transplanted iNPCs in Rag2 KO mice at 6 months post-transplantation. (a) Distribution of EGFP-positive iNPCs in the mouse hippocampus and their colocalization with NeuN immunostaining, indicating integration into neuronal regions (green: EGFP; red: NeuN; blue: DAPI). (b) Co-staining of EGFP with MAP2 and hNA demonstrates that iNPC-derived cells exhibit morphological features of mature neurons (arrows) (green: EGFP or MAP2; red: hNA; blue: DAPI). (c) Higher-magnification images showing colocalization of EGFP-positive cells with human neuronal marker (anti-hNA, arrows), indicating differentiation of iNPCs into human neurons (green: EGFP; red: hNA; blue: DAPI). (d) EGFP-positive neurites extend into the molecular layer of the dentate gyrus (dashed line) and colocalize with the postsynaptic marker PSD-95, suggesting synapse formation (green: EGFP; red: PSD-95; blue: DAPI). Panels a and b show higher-magnification images of the boxed regions.

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3.3. Behavior-induced Fos expression reveals functional integration of transplanted iNPCs

We next examined whether iNPC-derived neurons can be activated during learning. Entorhinal-DG circuit encodes novelty and contextual information [11]. Novel environment exploration or contextual fear conditioning can robustly induce DG neuron activation which can be recorded by the expression of the immediate early gene Fos. In WT mice transplanted with iNPCs for 1 month, both novel environment exploration and contextual fear conditioning induce sparse Fos expression in mouse DG neurons (Figure 3B, arrows). However, no Fos expression was detected in EGFP-positive iNPC-differentiated neurons (Figure 3B).

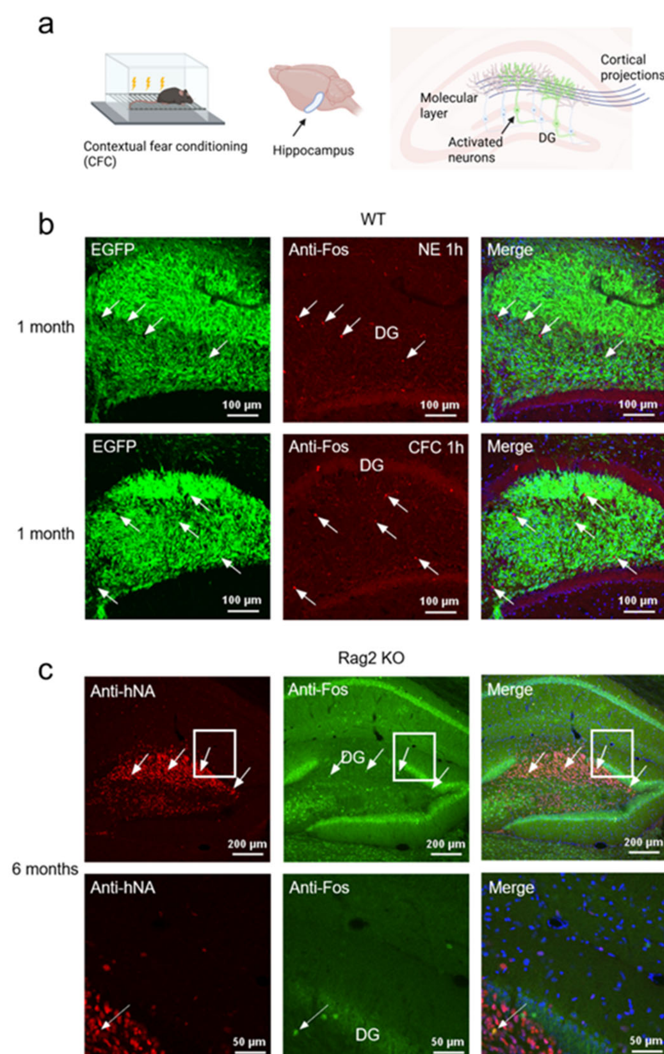

Figure 3. Behavior-induced Fos expression and functional integration of transplanted iNPCs. (a) Schematic diagram of the contextual fear conditioning (CFC) behavioral paradigm, the location of the hippocampus, and the entorhinal-hippocampal circuit involved in encoding contextual information. (b) Fos expression in WT mice 1 month after iNPC transplantation following 1 hour of novel environment exploration (NE) and 1 hour of contextual fear conditioning (CFC). Fos expression (red) and EGFP-positive iNPCs (green) are shown in the dentate gyrus (DG), with co-localization indicated by arrows (green: EGFP; red: Fos; blue: DAPI). (c) Fos expression in Rag2 KO mice 6 months after iNPC transplantation. Lower panels show higher-magnification images of the boxed regions from the previous panels, with co-localization of Fos and the human neuronal nuclear marker (hNA, red) in the dentate gyrus (DG). Co-localization is indicated by arrows (red: Fos; green: hNA; blue: DAPI).

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We next examined iNPC-translpanted Rag2 KO mice 6 months post-injection. Following contextual fear conditioning and a 1-hour interval for Fos expression, Fos-positive cells were detected in the dentate gyrus region (Figure 3C). Importantly, co-localization between Fos and hNA-positive transplanted neurons was observed (arrows), indicating that iNPC-derived neurons are activated by behavioral stimulation (Figure 3C). Together, these results demonstrate that although transplanted iNPCs do not exhibit functional responses at early time points in WT mice, long-term survival in Rag2 KO mice enables their differentiation and functional integration into hippocampal circuits.

4. CONCLUSION

The integration of xenografted human neurons or organoids with host organism is critical for the effectiveness of disease modeling or cell replacement therapy. In the brain, in addition to molecular and cellular integration for example synapse formation, their incorporation into functional network is also essential. We used endogenous Fos expression as an indicator of neuronal activation and found that iNPCs-differentiated neurons can incorporate into functional network of mouse hippocampus in response to learning-related behavior tasks.

Fos expression is strictly regulated by neuronal activity in the brain and is widely used to map brain regions that are involved in particular physiological or pathological conditions [17-21]. Tools based on Fos promoter have been developed to label neuronal populations and manipulate their activities to confirm their specific roles in various behavior tasks [10, 12, 13, 16]. Although we did not confirm the role of Fos-expression iNPC-differentiated neurons in memory encoding, the detection of their activation alone is a strong evidence that they can functionally integrated into local circuit of mouse hippocampus. We showed evidence that the basal level expression of Fos is suppressed in iNPC-differentiated neurons without external input (Figure 3B).

Dentate gyrus neurons receive projections from principal neurons at the second layer of entorhinal cortex through perforant pathway [22]. Fear conditioning stimulates entorhinal-hippocampal circuit to encode contextual information of an episodic memory [23]. Synapses formed between DG neuron dendrites and entorhinal neuron axonal projections are mostly in the molecular layer. We observed that the neurites of iNPC-differentiated neurons can also extend into DG molecular layer. The vast amount of DG neurons ensure separated encoding of similar/overlapped inputs from multiple contexts (pattern separation) [24]. In a few of iNPC-differentiated dendrites we detected PSD-95 signal in dendritic spine-like protrusions, suggesting mature synapses formed between iNPC-differentiated neurons and perforant pathway projections.

There are limitations of this study. Fos expression is an indirect measure of neuronal activity and does not reflect the realtime response ot DG neurons to novel environmental exploration. Our finding does not support a causal role of transplanted neurons in behavior induction. Future studies combining optogenetics, calcium imaging, or electrophysiological recordings will be required to determine the precise functional contribution of transplanted human neurons. In addition, long-term behavioral analyses may further elucidate their role in cognitive processes.

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