Cell Stem Cell, Volume *18*

Supplemental Information

Chemical Control of Grafted Human PSC-Derived Neurons in a Mouse Model of Parkinson's Disease

Yuejun Chen, Man Xiong, Yi Dong, Alexander Haberman, Jingyuan Cao, Huisheng Liu, Wenhao Zhou, and Su-Chun Zhang

Figure S1

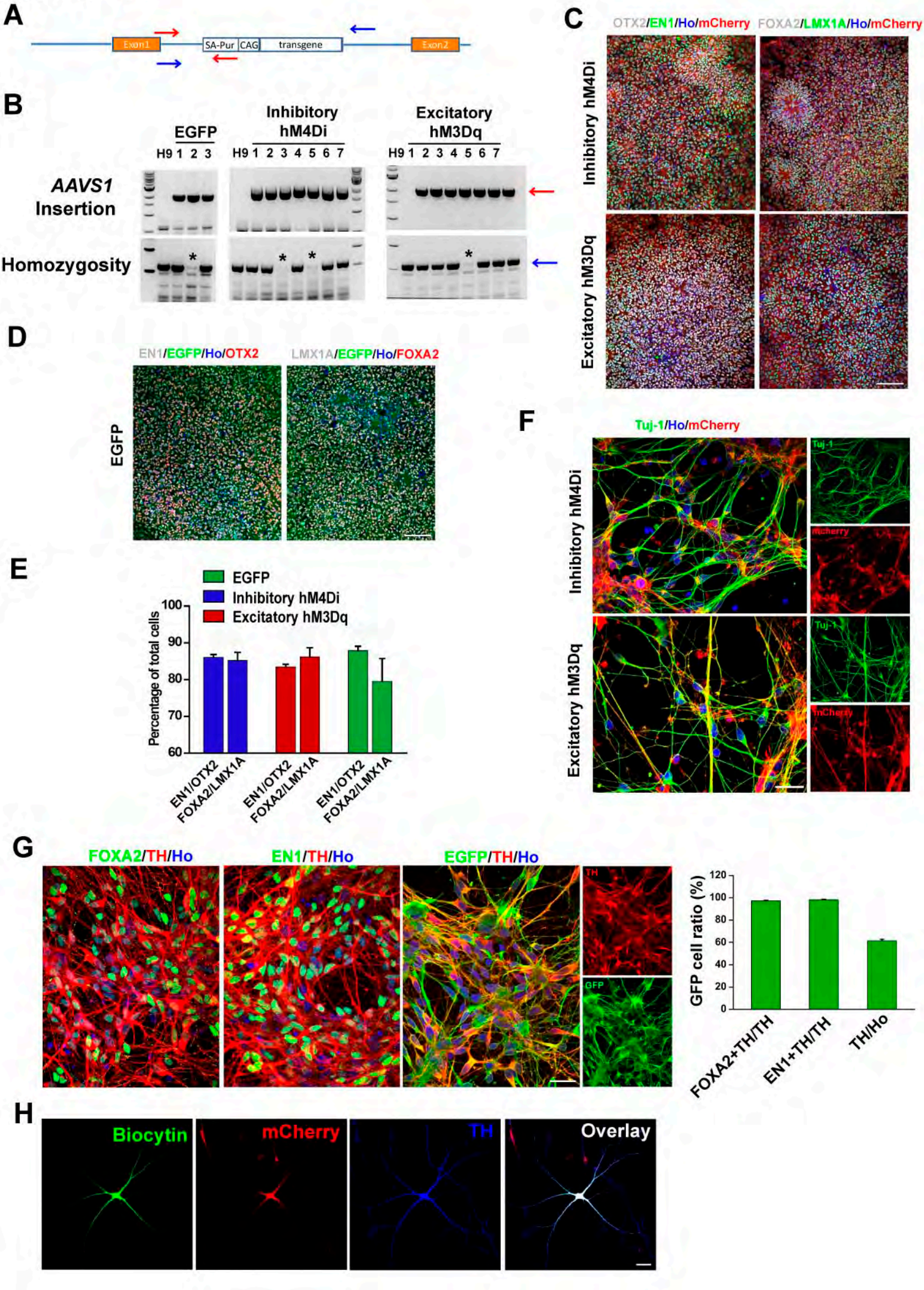
A
Exon1
SA-Pur
CAG
transgene
Exon2
B
EGFP
Inhibitory hM4Di
Excitatory hM3Dq
H9 1 2 3
H9 1 2 3 4 5 6 7
H9 1 2 3 4 5 6 7
AAVS1 Insertion
Homozygosity
C
OTX2/EN1/Ho/mCherry
FOXA2/LMX1A/Ho/mCherry
Inhibitory hM4Di
Excitatory hM3Dq
D
EN1/EGFP/Ho/OTX2
LMX1A/EGFP/Ho/FOXA2
EGFP
E
EGFP
Inhibitory hM4Di
Excitatory hM3Dq
Percentage of total cells
100
90
80
70
60
EN1/OTX2 FOXA2/LMX1A
EN1/OTX2 FOXA2/LMX1A
EN1/OTX2 FOXA2/LMX1A
F
Tuj-1/Ho/mCherry
Inhibitory hM4Di
Excitatory hM3Dq
Tuj-1
mCherry
G
FOXA2/TH/Ho
EN1/TH/Ho
EGFP/TH/Ho
TH
GFP
GFP cell ratio (%)
120
100
80
60
40
20
0
FOXA2+TH/TH
EN1+TH/TH
TH/Ho
H
Biocytin
mCherry
TH
Overlay

Figure S1: Immunostaining of differentiated mDA neurons derived from EGFP, inhibitory hM4Di, or excitatory hM3Dq hESCs. Related to Figure 1

(A) Genotyping strategy for *AAVS1* targeting of the transgene expressing cassette. The red arrows and blue arrows indicate PCR primers for *AAVS1* locus insertion or homozygosity, respectively. (B) PCR genotyping of EGFP-, inhibitory hM4Di-, or excitatory hM3Dq-expressing hESC clones. The expected PCR products for correctly targeted *AAVS1* locus are ~2000bp (red arrows). These clones underwent a further homozygosity assay. Clones with the PCR products of ~650 bp are heterozygous (blue arrow), and those clones without PCR products are homozygous (black asterisk). (C-E) Immunostaining (C-D) and quantification (E) for mDA neuron progenitor markers at day 20 of differentiation from inhibitory hM4Di (C), excitatory hM3Dq (C), or EGFP hESCs (D). Ho, Hoechst. Scale bar = 100μm. (F) Immunostaining for Tuj1 and mcherry at day 42. Scale bar = 25μm. (G) Immunostaining and quantification of mDA neuron differentiation cultures from EGFP hESCs at day 42. Ho, Hoechst. TH, tyrosine hydroxylase. Scale bar = 25μm. (H) Immunostaining shows the expression of TH and mCherry in the biocytin-labeled, recorded neuron. Scale bar = 50μm. TH, tyrosine hydroxylase.

Figure S2

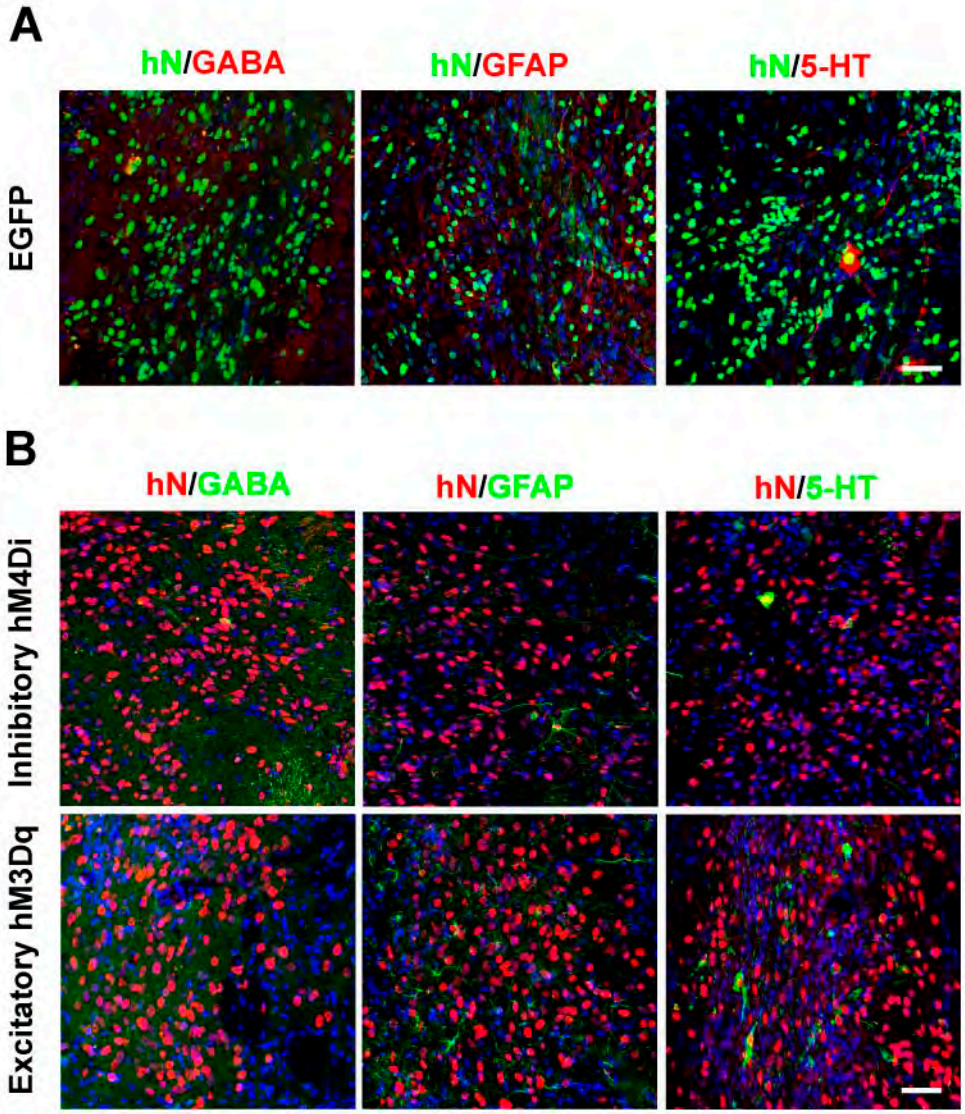
A
hN/GABA
hN/GFAP
hN/5-HT
EGFP
B
hN/GABA
hN/GFAP
hN/5-HT
Inhibitory hM4Di
Excitatory hM3Dq

Figure S2. Immunohistochemistry of cell types in grafts. Related to Figure 2.
(A-B) Immunostaining images show GABA-positive, GFAP-positive and 5-HT positive cells in the human graft from EGFP (A), hM4Di and hM3Dq group (B). Scale bar = 25 μm. hN, human nuclei.

Figure S3

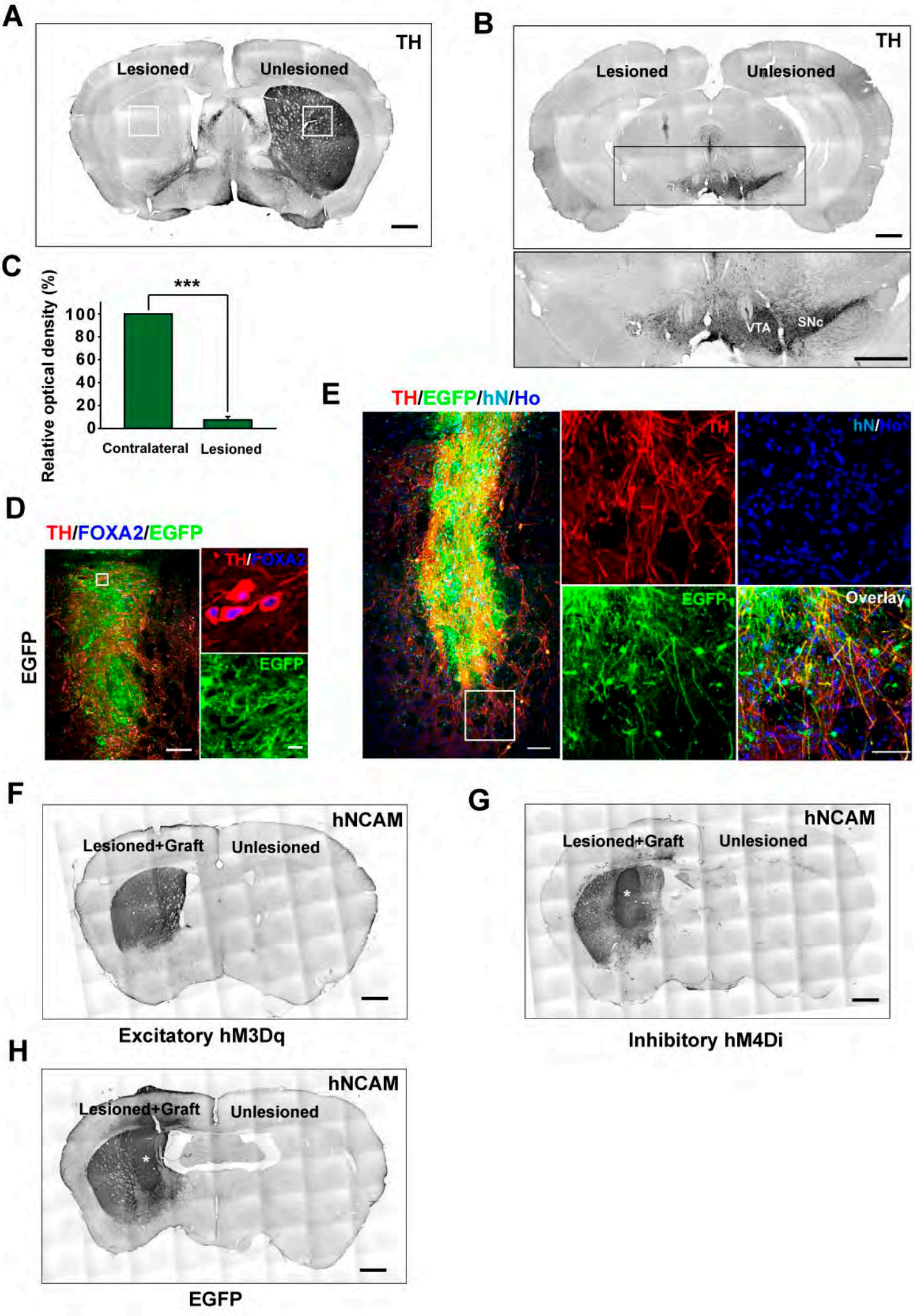
A
TH
Lesioned
Unlesioned
B
TH
Lesioned
Unlesioned
VTA
SNc
C
***
Relative optical density (%)
100
80
60
40
20
0
Contralateral
Lesioned
D
TH/FOXA2/EGFP
EGFP
TH/FOXA2
EGFP
E
TH/EGFP/hN/Ho
TH
hN/Ho
EGFP
Overlay
F
hNCAM
Lesioned+Graft
Unlesioned
Excitatory hM3Dq
G
hNCAM
Lesioned+Graft
Unlesioned
Inhibitory hM4Di
H
hNCAM
Lesioned+Graft
Unlesioned
EGFP

Figure S3. Innervation of PD mouse brain by human grafts. Related to Figure 2.
(A-B) TH staining shows depletion of TH+ fibers in the lesioned site of striatum (A) and loss of TH neurons in the lesioned substantia nigra compacta (SNc) (B) 6 month after unilateral 6-OHDA lesion. VTA, ventral tegmental area. Boxed area in (B) is amplified below. Scale bar = 500μM. (C) TH optical density in the areas corresponding to white boxes in (A). Student's *t*-test, ***$P<0.001$ (n = 6). (D) Immunostaining shows that the grafted TH positive cells co-express FOXA2 as well as EGFP at 6 months after transplantation. This is a composite image of two separate fields. Boxed area is amplified at right.. Scale bar = 100μM, left panel; Scale bar = 10μM, right panel. (E) Immunostaining images show outgrowth of fibers from the EGFP graft and presence of fibers in the host tissue. This is a composite image of two separate fields. Boxed area is amplified at right. These TH positive fibers co-expressed EGFP. The absence of hN staining at right panel indicates host cells. Scale bar = 100μM, left panel; Scale bar = 50μM, middle and right panel. (F-H) Immunohistochemistry of hNCAM reveals that mDA neuron-enriched human graft 6 months after intrastriatal transplantation. (F) Brain slice from hM3Dq graft away from the graft core. (G) hM4Di graft. (H) EGFP graft. White asterisk indicates graft sites. Scale bar = 500μm.

Figure S4

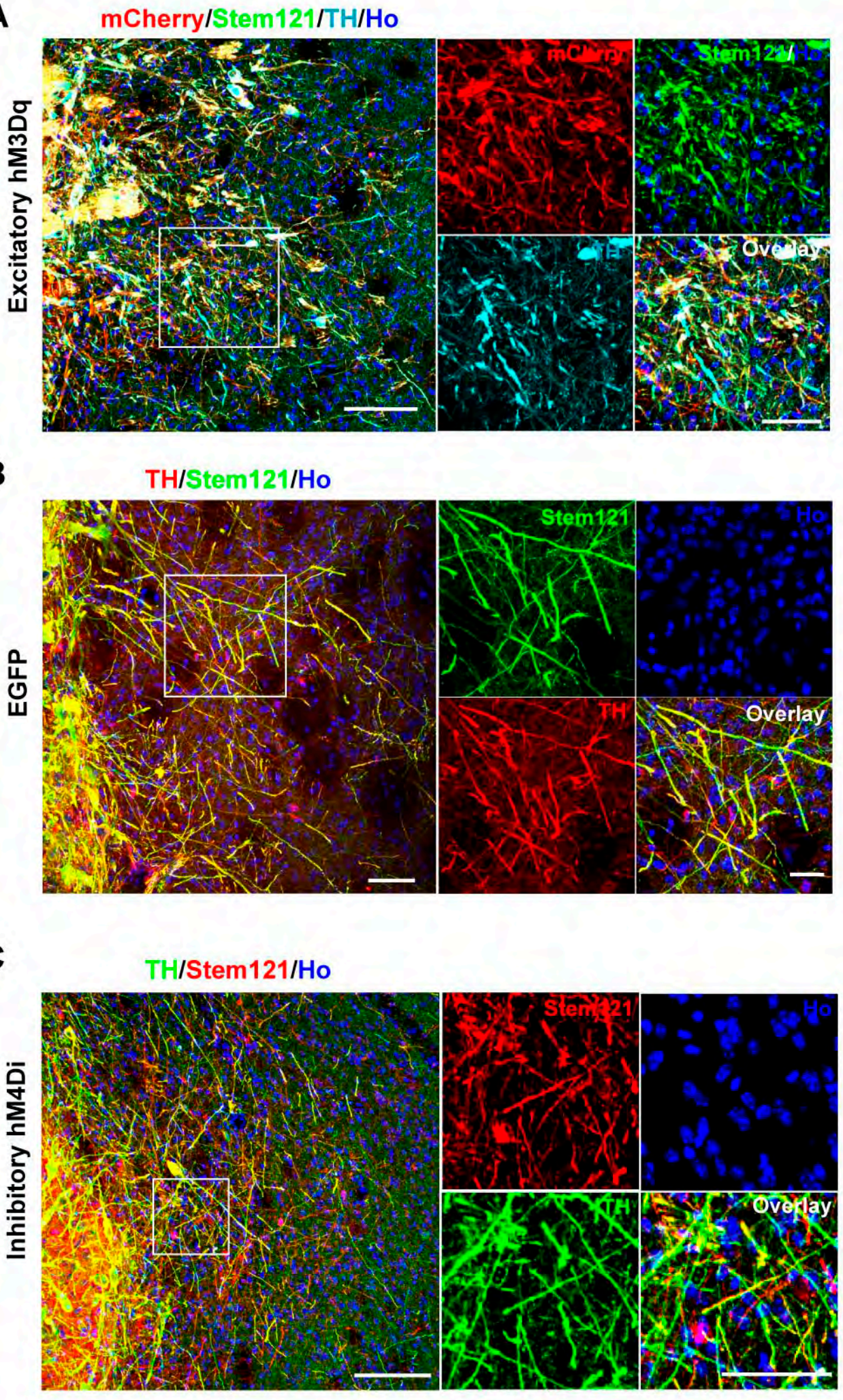
A
mCherry/Stem121/TH/Ho
Excitatory hM3Dq
mCherry
Stem121/Ho
TH
Overlay
B
TH/Stem121/Ho
EGFP
Stem121
Ho
TH
Overlay
C
TH/Stem121/Ho
Inhibitory hM4Di
Stem121
Ho
TH
Overlay

Figure S4. Fiber outgrowth from human grafts. Related to Figure 2.
Immunostaining images show outgrowth of fibers from hM3Dq graft (A), EGFP graft (B), and hM4Di graft (C). Boxed areas in are amplified at right. These stem121 positive human fibers co-expressed TH (A, B, C) or mCherry (A). Scale bar = 50μM, left; Scale bar = 25μM, right.

Movie S1-S4. Effects of CNO on spontaneous rotation. Related to Figure 4.
Spontaneous rotation test the groups grafted with mDA neurons expressing inhibitory hM4Di (Movie S1 and Movie S2), or excitatory hM3Dq (Movie S3 and Movie S4). The videos show the preferential rotation of the same mice in each group with CNO (Movie S2 and Movie S4) or without CNO (Movie S1 and Movie S3) treatment.

Supplemental Experimental Procedures

Construction of donor plasmid

Human codon-optimized Streptococcus pyogenes wild-type Cas9 (Cas9-2A-GFP) and sgRNA T2 were obtained from Addgene (plasmid #44719, plasmid#41818) (Ding et al., 2013; Mali et al., 2013). To generate AAVS1-pur-CAG-EGFP donor plasmid, we replaced the hrGFP gene in the AAVS1-pur- CAG-hrGFP plasmid (Addgene plasmid #52344) (Qian et al., 2014) with EGFP gene and inserted woodchuck hepatitis post-transcriptional regulatory element (WPRE) and human growth hormone (hGH) Poly A into the 3' terminal of EGFP gene to obtain AAVS1-pur-CAG-EGFP donor plasmid. We next amplified hM4Di-mCherry or hM3Dq-mCherry cDNA by PCR from AAV-DIO-hM4Di-mCherry plasmid or AAV-DIO-hM3Dq-mCherry plasmid (gifts from Dr. Bryan L. Roth), respectively. hM4Di-mCherry or hM3Dq-mCherry was inserted into the AAVS1-pur-CAG-EGFP donor plasmid to replace EGFP to get the AAVS1-pur-CAG-hM4Di-mCherry or AAVS1-pur-CAG-hM3Dq-mCherry donor plasmid.

Generation of midbrain dopamine neurons

EGFP- and DREADD-expressing hESCs (1 day after passaging) on MEF were cultured in the neural induction medium consisting of Dulbecco's modified Eagle's medium/F12, N2 supplement, nonessential amino acids (Life technologies) in the presence of 2 μM SB431542 (Stemgent), 2 μM DMH1 (Torcris), SHH (C25II, 500 ng/ml, R&D Systems) and CHIR99021 (0.4 μM, Stemgent) for 8 days. On day 9, individual colonies of epithelial cells were gently blown off and expanded as floating clusters in suspension in the neural induction medium containing SAG (2 μM, Calbiochem), SHH (C25II, 100 ng/ml) and CHIR99021(0.4 μM) for 4 days (D9-12).

Then the progenitors in suspension were cultured in the neural induction medium containing SAG (0.5 µM, Calbiochem) and FGF8b (100 ng/ml, PeproTech) for another week (D13-19). Progenitors will be kept in FGF8b (100 ng/ml) and SHH (20 ng/ml) till transplantation at day 32. For in vitro analysis, the neurospheres were dissociated by ACCUTASE (Innovative Cell Technologies) on day 35 and plating onto glass coverslips that were coated with matrigel (BD Biosciences) in the neurobasal medium supplemented with 1% N2 (Life Technologies), 2% B27 (Life Technologies), brain-derived neurotrophic factor (10 ng/ml, PeproTech), glial-derived neurotrophic factor (10 ng/ml, PeproTech), ascorbic acid (200 µM, Sigma-Aldrich), cAMP (1 µM, Sigma-Aldrich), transforming growth factor β3 (1 ng/ml, R&D Systems) and Compound E (1 µM, Calbiochem).

Immunocytochemistry and Quantification

Cells on coverslips were fixed in 4% paraformaldehyde for 20 min and rinsed with PBS. Cells were treated with 0.2% Triton for 10 min followed by 10% donkey serum for 1 hour before incubation with primary antibodies overnight at 4℃. Cells were then incubated for 1h at room temperature with fluorescently conjugated secondary antibodies (Life Technologies). The nuclei were stained with Hoechst (Ho) (Sigma-Aldrich). Images were collected with a Nikon A1R-Si laser-scanning confocal microscope (Nikon, Tokyo, Japan). Fields (>6 each coverslip) were randomly selected and the neuronal cells and total cells (Hoechst-stained) were counted using ImageJ software. Data were replicated three times in each cell lines and were expressed as mean ± SEM. For immunohistochemistry on brain slices, animals were sacrificed at 6-7 months after transplantation or 6 months after 6-OHDA lesion with an overdose of pentobarbital (250 mg/kg, i.p.) and perfused with 0.9% saline solution followed by

4% ice-cold phosphate-buffered paraformaldehyde (PFA). The brains were removed and immersed sequentially in 20% and 30% sucrose until sunk. Coronal sections were cut on a microtome at a thickness of 30μm and stored at -20 °C in cryoprotectant solution. For DAB staining, free-floating sections were incubated with primary antibody (Supplemental table1) at 4ºC for 1-2 nights. After the unbound primary antibodies were removed, sections were incubated with corresponding secondary antibody for 1h at room temperature. Then sections were mounted by Fluoromount-G^{TM} (Southern Biotech). For DAB staining, Sections were incubated with biotinylated secondary antibodies for 1h followed by avidin-biotin peroxidase for 1h at room temperature. Immunoreactivity was visualized with DAB staining kit (Vector Laboratories). Detailed information about primary antibodies is given in Supplemental Experimental Procedures.

Whole-Cell Patch-Clamp and Brain Slice Recording

Acute slices (320 μm thick) were prepared from recovered animals at 6 months post-transplantation using a vibratome (Leica VT1000S) and placed in ice-cold cutting solution containing (in mM): 100 glucose, 75 NaCl, 26 $NaHCO_3$, 2.5 KCl, 2 $MgCl_2$-$6H_20$, 1.25 NaH_2PO_4-$6H_20$, and 0.7 $CaCl_2$. Slices were allowed to recover in the solution for 30 minutes at 30°C and then transferred to recording aCSF containing (in mM): 119 NaCl, 26.2 $NaHCO_3$, 10 glucose, 2.4 $CaCl_2$, 1.8 KCl, 1.2 $MgCl_2$-$6H_20$, and 1.0 NaH_2PO_4-$6H_20$. The temperature of recording chamber was maintained at 30°C perfused with aCSF saturated with 95% O_2/5% CO_2.

Electrodes were filled with a solution containing 140 mM K-gluconate, 0.1 mM CaCl2, 2 mM MgCl2, 1 mM EGTA, 2 mM ATP K2, 0.1 mM GTP Na3 and 10 mM HEPES (pH = 7.25). The resistance of the electrodes was 4–6MΩ. Voltage and current

signals were recorded with an Axopatch 700B amplifier (Axon) connected to a Digidata1322A interface (Axon). The data was digitized and stored on disks using pClamp (version 9; Axon). Resting membrane potential and action potentials were recorded under the current clamp mode. sEPSCs were recorded in sweeps of 10 s at a holding potential of −70 mV under the voltage clamp mode in the presence of picrotoxin (10 mM). The series resistance (Rs) was monitored by measuring the instantaneous current in response to a 5-mV voltage step command. Series resistance compensation was not used, but cells where Rs changed by >15% were discarded.

Antibodies

Primary antibodies were used as detailed: mouse anti-EN1 (1:500; DSAB 4G11); goat anti-FOXA2 (1:500; Santa Cruz sc-6554); rabbit anti-LMX1A (1:500; Gift from Dr. M.S. German); rat anti-mCherry (1:1000; Life Technology M11217); mouse anti-TUJ-1 (1:6000; Sigma T8660); rabbit anti-TH (1:500; Pel-Freez Biologicals P40101); mouse anti-TH (1:5000; Sigma T1299); mouse anti-Human Nuclei (1:400; Millipore MAB1281); mouse anti-hNCAM (1:1000; Santa Cruz sc-106); mouse anti-Stem121 (1:500; Clontech Y40410); goat anti-OTX2 (1:500; R&D AF1979); goat anti-EGFP (1:1000; Abcam ab5450); rabbit anti-5-HT (1:4000; Sigma S5545); rabbit anti-GABA (1:5000; Sigma A2052); rabbit anti-GFAP (1:5000; DAKO Z0334).

Animal surgery and cell transplantation

To create a PD model, adult SCID mice (8-10 weeks of age) were anesthetized with 1%–2% isoflurane mixed in oxygen and received a stereotaxic injection of 1µl 6-OHDA (3 mg/ml, in saline with 1% ascorbic acid) directly into the left substantial nigra on a coordinate of anterior-posterior [AP] = - 2.9 mm, lateral [L] = -1.1 mm, and vertical [V] = -4.5 mm. Successfully lesioned mice (>6 amphetamine-induced

rotations/min) were selected for transplantation (Grealish et al., 2010; Heuer et al., 2012; Iancu et al., 2005). The animals were randomized and received inhibitory hM4Di-, excitatory hM3Dq-, or EGFP-expressing mDA transplantation. About 2×10^5 cells in 2 μl artificial cerebrospinal fluid (aCSF) containing B27, 20 ng/ml BDNF were injected into the left striatum at the following coordinates (AP = +0.6 mm, L = -1.8 mm, V = -3.2 mm) 4 weeks after 6-OHDA lesion surgery.

Behavioral Tests

Rotation test

Amphetamine-induced rotations were carried out before transplantation and 3, 4, 5 months after transplantation. Rotation behavior in mice was recorded 10 min after intraperitoneal injection of D-amphetamine (Sigma, 5 mg/kg) and recorded with video system for 90 min. For spontaneous rotation test, animals were recorded for 90 min after intraperitoneal injection of CNO (Biomol International, 4 mg/kg for inhibitory hM4Di group, 1.2 mg/kg for excitatory hM3Dq group) or saline for 20 min. For testing the involvement of dopamine receptors, The selective D1 antagonist SCH-23390 (Sigma-Aldrich, 0.1 mg/kg) and selective D2 antagonist Raclopride (Sigma-Aldrich, 1 mg/kg) were intraperitoneally injected together 5 min before CNO treatment. Rotation was recorded for 90 min after CNO injection for 20 min. Videos were analyzed by the investigators blind to the groups. One full-body turn was counted as a value of 1. The rotation data were presented as the average ipsilateral net number of rotations per minute or percentage of ipsilateral rotation during 90 min.

Cylinder test

Individual animal was placed in a glass cylinder after CNO injection for 40 min and recorded by a camera system for 3min. The ipsilateral and contralateral paw touches

to the wall of the cylinder were counted. The minimum touch number is 20 in total 3 min video. Videos were analyzed by the investigators blind to the groups. The data are expressed as the percentage of ipsilateral touches to total touches.

Rotarod Test

An accelerating Rotarod (Med Associates Instruments) was used to test motor coordination. All animals were pre-trained for two days in order to reach a stable performance. On day-1, mice were trained on a rotating rod that accelerated from 2 per minute (rpm) to 20 rpm in a period of 300s for three times. On day-2, mice were trained on rod accelerated from 3 per minutes (rpm) to 30 rpm twice, and from 4 per minute (rpm) to 40 rpm once, in a period of 300s. The test was performed from the third day on a rotating rod that accelerated from 4 per minute (rpm) to 40 rpm in a period of 300 s after CNO injection for 40 min. The period of time the mouse stayed on the rod was monitored. The average duration from three repeated tests of each animal was used for data analyzed.

Stereological Analysis of Grafts

Serial sections (30 μm, every 6^{th} section from the graft) were taken for stereological quantification of transplanted human cells positive for TH. The number of grafted TH+ neurons were counted under a ×20 objective of a Zeiss fluorescence scope in fields chosen by the StereoInvestigator software (MicroBrightField, Inc) as described previously (Liu et al., 2013). The total human cells within the whole graft were estimated by the Stereo Investigator software. Data are presented as mean ± SEM.

REFERENCES

Ding, Q., Regan, S.N., Xia, Y., Oostrom, L.A., Cowan, C.A., and Musunuru, K. (2013). Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. Cell Stem Cell *12*, 393-394.

Grealish, S., Mattsson, B., Draxler, P., and Bjorklund, A. (2010). Characterisation of behavioural and neurodegenerative changes induced by intranigral 6-hydroxydopamine lesions in a mouse model of Parkinson's disease. Eur J Neurosci *31*, 2266-2278.

Heuer, A., Smith, G.A., Lelos, M.J., Lane, E.L., and Dunnett, S.B. (2012). Unilateral nigrostriatal 6-hydroxydopamine lesions in mice I: motor impairments identify extent of dopamine depletion at three different lesion sites. Behav Brain Res *228*, 30-43.

Iancu, R., Mohapel, P., Brundin, P., and Paul, G. (2005). Behavioral characterization of a unilateral 6-OHDA-lesion model of Parkinson's disease in mice. Behav Brain Res *162*, 1-10.

Liu, Y., Weick, J.P., Liu, H., Krencik, R., Zhang, X., Ma, L., Zhou, G.M., Ayala, M., and Zhang, S.C. (2013). Medial ganglionic eminence-like cells derived from human embryonic stem cells correct learning and memory deficits. Nat Biotechnol *31*, 440-447.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. (2013). RNA-guided human genome engineering via Cas9. Science *339*, 823-826.

Qian, K., Huang, C.T., Chen, H., Blackbourn, L.W.t., Chen, Y., Cao, J., Yao, L., Sauvey, C., Du, Z., and Zhang, S.C. (2014). A simple and efficient system for regulating gene expression in human pluripotent stem cells and derivatives. Stem Cells *32*, 1230-1238.