Supplementary Data

Supplementary Methods

Behavioral testing

Behavioral testing was conducted 1 week before transplantation and 2, 4, 8, and 12 weeks after transplantation. Observers were blinded to the treatment groups during testing and *post hoc* analysis.

Apomorphine-induced rotations. Mice were placed into acrylic cylinders (15.24 cm diameter × 25.4 cm height) and recorded from above with a video camera for later analysis. Mice were allowed to habituate to the cylinder for 5 min before injecting apomorphine hydrochloride (0.5 mg/kg, s.c.),[S1] and recording for a period of 30 min, counting the number of full body ipsilateral and contralateral rotations relative to the lesioned hemisphere.

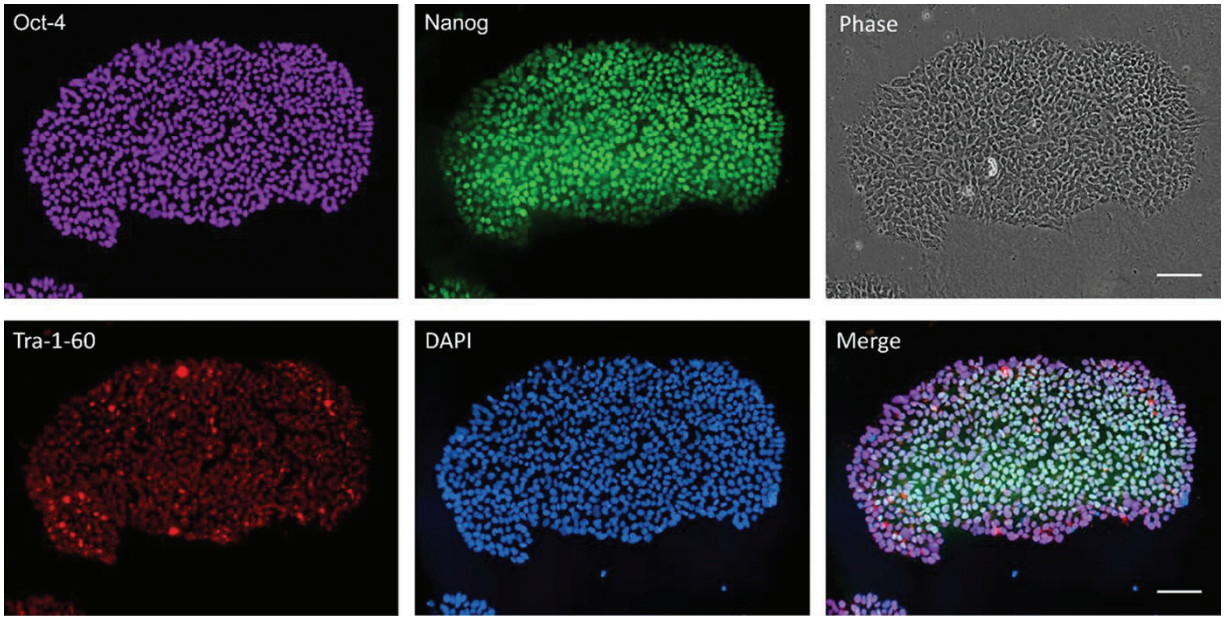
Cylinder test. Mice were allowed to explore their surroundings within an acrylic cylinder for ~5 min while recording from the side with a video camera. The cylinder was placed next to two angled mirrors to visualize forelimb movements from all angles.[S2,S3] Forelimb asymmetry was assessed by counting the independent, weight-bearing ipsilateral, contralateral, and simultaneous paw touches (relative to the lesioned hemisphere) on the cylinder wall when rearing and on the floor when landing. Asymmetry scores were calculated by using the formula: [(contra+½ both) divided by (ipsi+contra+both)] × 100, averaging the scores for rearing and landing.[S2]

Rotarod. Mice were trained on a Panlab Rotarod LE8205 (Harvard Apparatus) for three consecutive days (three trials per day at 4, 10, and 15 rpm respectively; 120 s maximum per trial). Latency to fall was recorded for three trials at a speed of 25 rpm (300 s maximum per trial). Mice were allowed to rest in their home cages for at least 5 min between trials to reduce stress and fatigue.

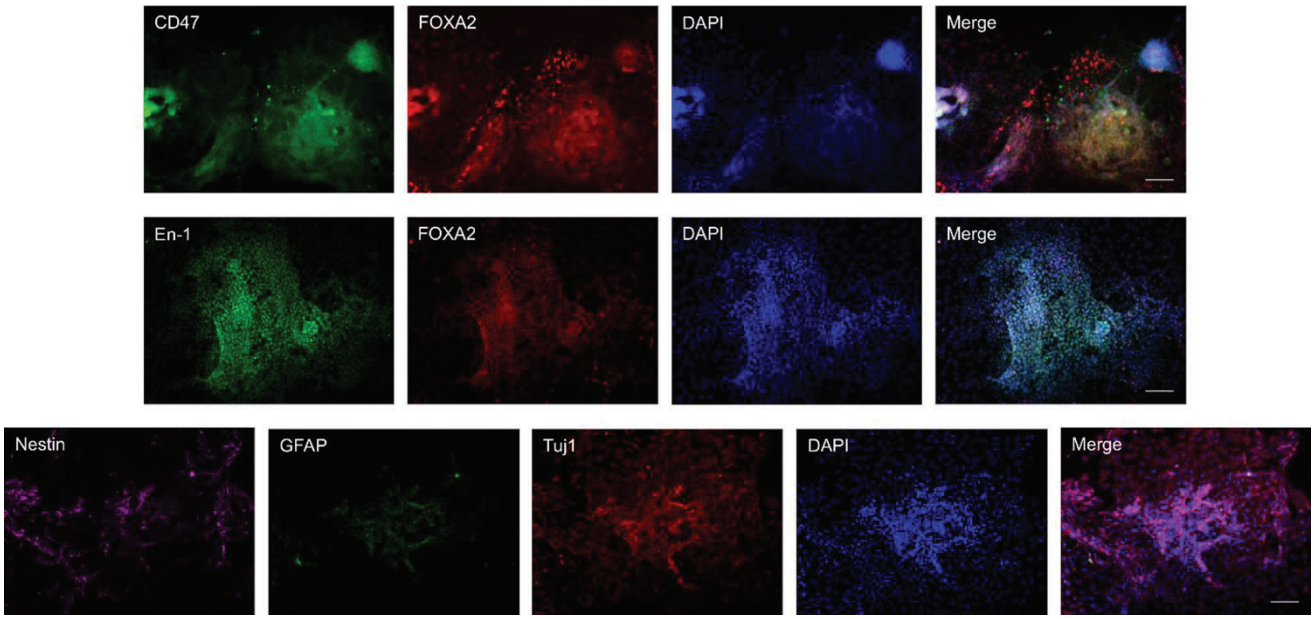
Tissue processing and immunohistochemistry

At 13 weeks post-transplantation, mice were deeply anesthetized with Euthasol and transcardially perfused with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.4. The brains were extracted, post-fixed in 4% PFA overnight, and cryoprotected in 30% sucrose for 3–5 days. Coronal sections (40 µm) were prepared by sectioning on a cryostat after embedding in Tissue-Tek O.C.T. Compound (VWR) and stored in PBS at 4°C. Cells cultured *in vitro* were fixed in 4% PFA for 15 min at room temperature and washed three times in PBS. The fixed samples (cells cultured *in vitro* or free-floating brain slices) were blocked/permeabilized in PBS containing 10% goat serum, 1% bovine serum albumin, and 0.1% Triton X-100 (MilliporeSigma) for 1 h at room temperature.

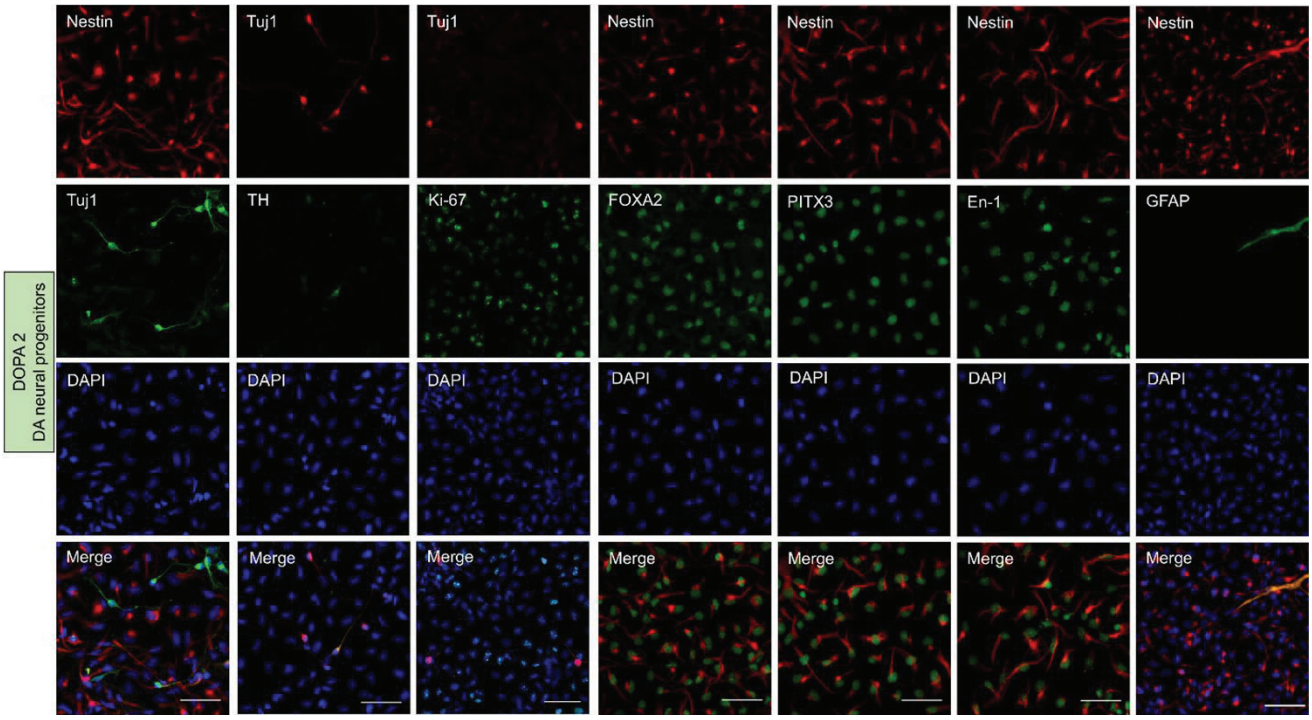
The samples were incubated with primary antibodies diluted in blocking buffer (full list shown in Supplementary Table S1) overnight at 4°C. Appropriate Alexa Fluor-conjugated secondary antibodies (1:500 dilution; ThermoFisher Scientific) were applied for 1 h at room temperature, and nuclei were counterstained with Hoechst 33342 (ThermoFisher Scientific). The samples were washed at least three times with PBS after staining. Brain slices were mounted on glass slides and coverslips with Prolong Gold Anti-Fade reagent (ThermoFisher Scientific) before imaging. Transplanted cell survival was quantified by using the three-dimensional (3D) Object Counter[S4] in Fiji/ImageJ after processing images with the 3D Spot Segmentation plugin.[S5] Quantification of cells expressing huNu and tyrosine hydroxylase was performed on every fifth coronal brain section (40 µm thick) throughout the striatum where a graft was identifiable.



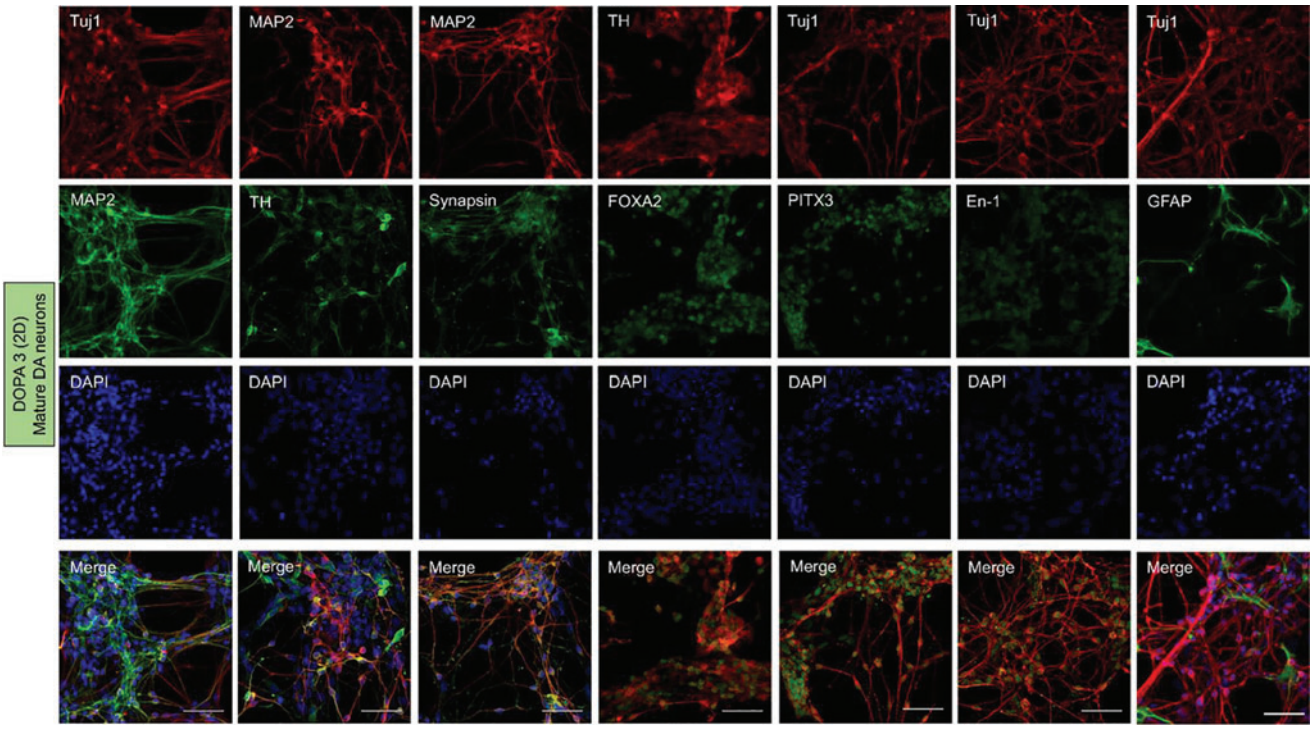
SUPPLEMENTARY FIG. S1. *In vitro* immunocytochemical staining of pluripotency markers in iPSCs (day −1 of differentiation). Scale bar: 100 µm.



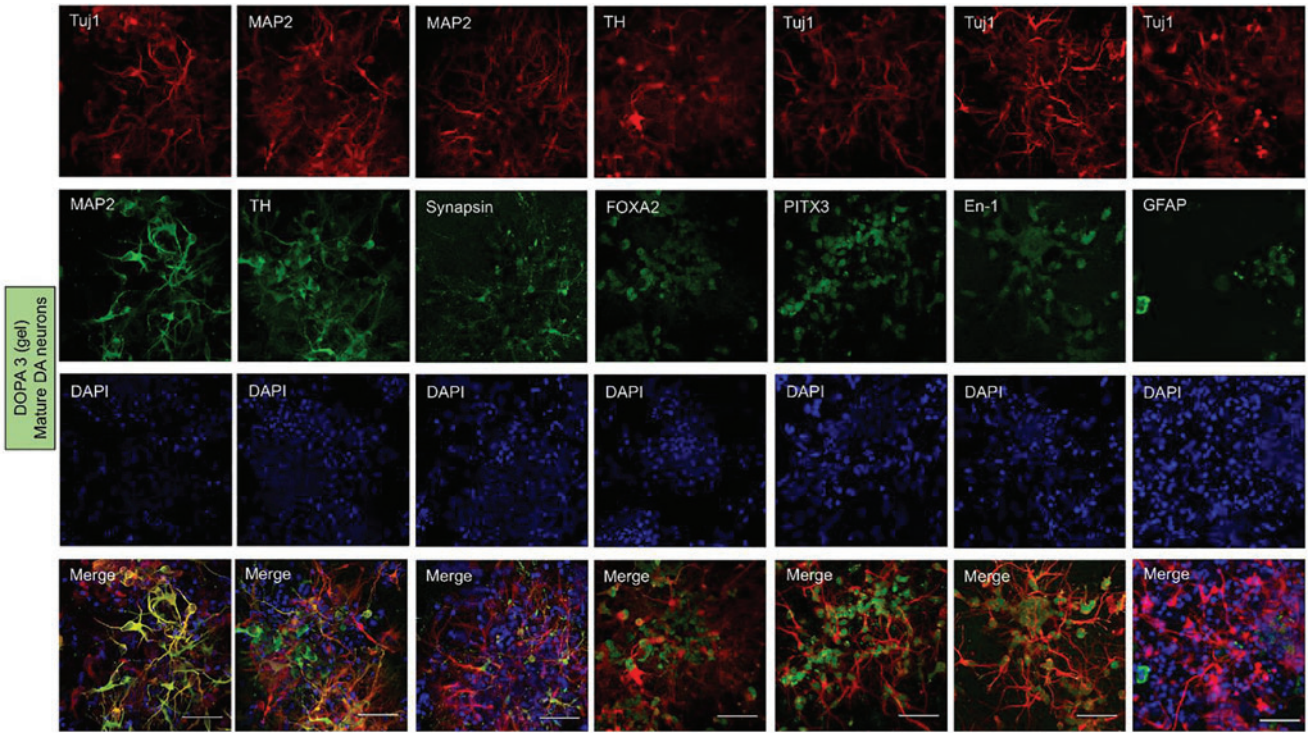
SUPPLEMENTARY FIG. S2. Immunocytochemical staining of late-stage DOPA1 cells 24 h after plating on a TCPS dish. Scale bar: 100 μm.



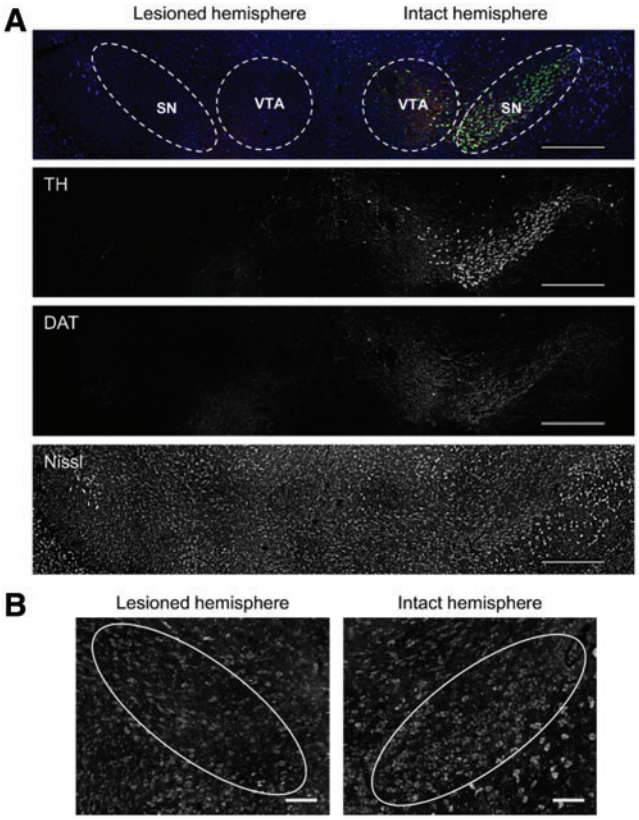
SUPPLEMENTARY FIG. S3. Immunocytochemical staining (split images) of DA neural progenitors (DOPA2) shown in Figure 2, displaying the following markers: Nestin: neural stem/progenitor, Tuj1 (β-III tubulin): neuron, TH: DA neuron, Ki-67: proliferative cells, FOXA2: midbrain DA neuron, PITX3: midbrain DA neuron, En-1 (Engrailed-1): midbrain DA neuron, GFAP: astrocyte, DAPI: nucleus. Scale bar: 50 μm. TH, tyrosine hydroxylase.



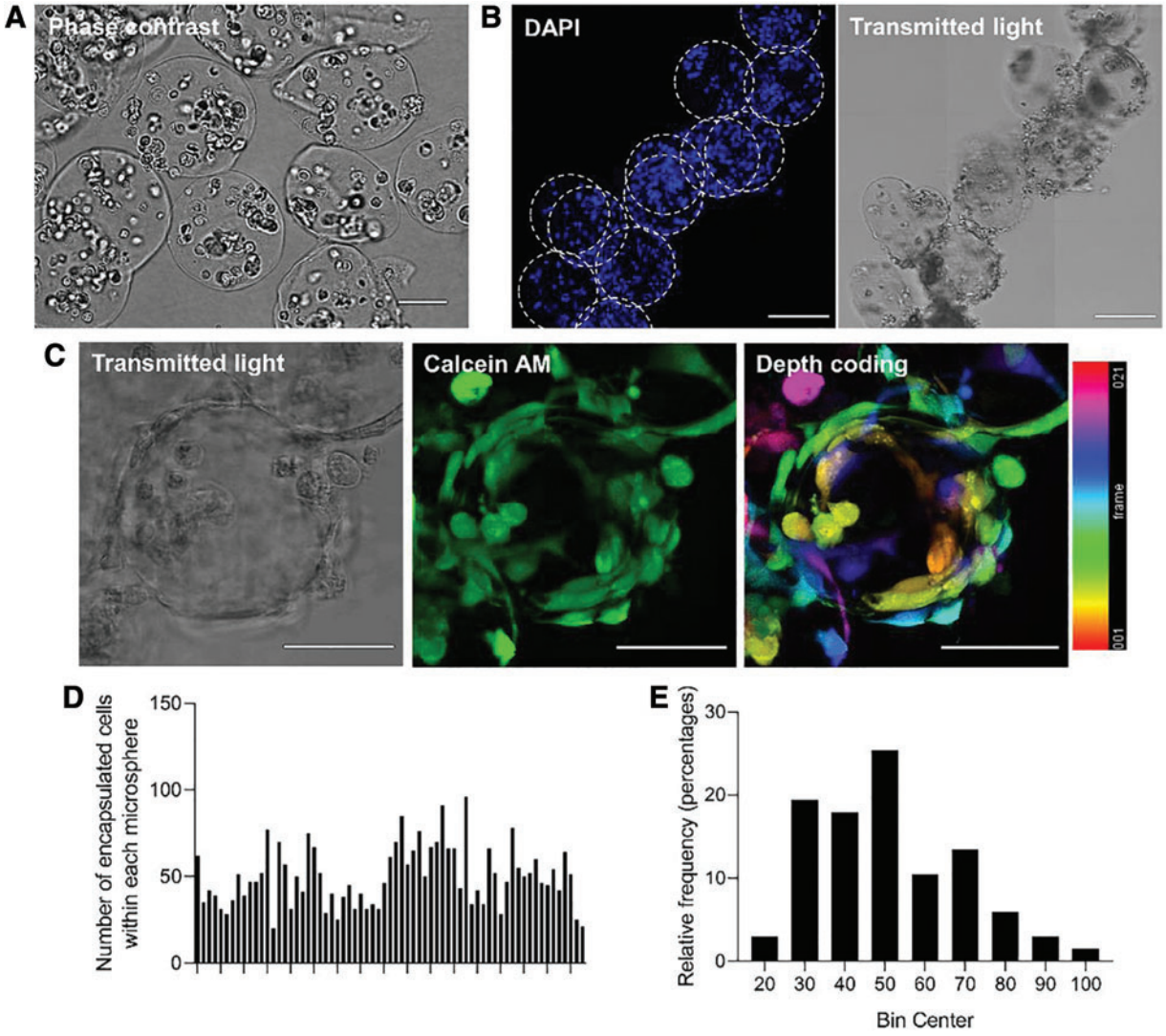
SUPPLEMENTARY FIG. S4. Immunocytochemical staining (split images) of 2D mature DA neurons shown in Figure 2, displaying the following markers: Tuj1 (β-III tubulin): neuron, MAP2: mature neuron, TH: DA neuron, Synapsin: synaptic vesicle, FOXA2: midbrain DA neuron, PITX3: midbrain DA neuron, En-1 (Engrailed-1): midbrain DA neuron, GFAP: astrocyte, DAPI: nucleus. Scale bar: 50 μm. 2D, two-dimensional.



SUPPLEMENTARY FIG. S5. Immunocytochemical staining (split images) of 3D-encapsulated mature DA neurons shown in Figure 2, displaying the following markers: Tuj1 (β-III tubulin): neuron, MAP2: mature neuron, TH: DA neuron, Synapsin: synaptic vesicle, FOXA2: midbrain DA neuron, PITX3: midbrain DA neuron, En-1 (Engrailed-1): midbrain DA neuron, GFAP: astrocyte, DAPI: nucleus. Scale bar: 50 μm. 3D, three-dimensional.



SUPPLEMENTARY FIG. S6. Immunohistochemical staining of the substantia nigra (SN) and ventral tegmental area (VTA) within 6-OHDA unilaterally lesioned mice. **(A)** TH, DAT, and Nissl staining of the SN VTA area. Scale bar: 500 µm. **(B)** Higher magnification of Nissl staining of the SN in the lesioned and intact hemisphere. Scale bar: 100 µm.



SUPPLEMENTARY FIG. S7. Quantification of cells encapsulated within RADA16-I microspheres. **(A)** Phase-contrast image of microspheres ~30 min after encapsulation. Scale bar: 50 μm. **(B)** Maximum intensity projection (Z-stack) of a cluster of microspheres stained with DAPI and the corresponding transmitted light image to illustrate position of the microspheres. *White dotted lines* indicate microsphere position. Scale bar: 100 μm. **(C)** Transmitted light image showing microsphere position; Maximum intensity projection (Z-stack) of Calcein-AM-stained microspheres; Temporal color-coding of Calcein-AM staining: color indicates Z-position (depth) of the cells within the microsphere. Scale bar: 50 μm. **(D)** Frequency distribution of cell counts within individual microspheres. **(E)** Histogram of the frequency distribution of cell counts within individual microspheres. Mean: 49.9701, standard deviation: 17.2925, standard error: 2.11261, lower 95% CI of mean: 45.7522, upper 95% CI of mean: 54.1881 ($n = 67$ microspheres). AM, acetoxymethyl; CI, confidence interval.

Supplementary Table S1. List of Primary Antibodies

Antibody	*Company*	*Catalog number*	*Dilution*
β-III tubulin (Tuj1)	Covance	MMS-435P	1:1000
MAP2	BD	556320	1:500
TH	MilliporeSigma	AB152	1:1000
TH	MilliporeSigma	MAB318	1:200
TH	MilliporeSigma	AB9702	1:1000
Nestin	abcam	MAB5326	1:100
Synapsin	Gift from the lab of Dr. Thomas Südhof	E028	1:3000
Human-specific cytoplasm (STEM121)	Takara Bio	STEM121	1:100
FOXA2	Santa Cruz	sc-101060	1:100
DAT	MilliporeSigma	MAB369	1:1000
PITX3	MilliporeSigma	AB5722	1:200
En-1	MilliporeSigma	AB5732	1:500
GFAP	Dako	Z033429	1:1000
Human-specific nuclei	MilliporeSigma	MAB1281	1:100
NCAM	Santa Cruz	sc-59864	1:100
Ki-67	abcam	ab15580	1:200
Oct-4	ThermoFisher Scientific	A13998	1:1000
Tra-1-60	MilliporeSigma	MAB4360	1:250
Nanog	MilliporeSigma	AB9220	1:500
CD47	ThermoFisher Scientific	14-0479-82	1:100
Collagen I	abcam	ab34710	1:500
Collagen IV	abcam	ab748	1:40
Fibronectin	Santa Cruz	sc-9068	1:100
Laminin	MilliporeSigma	L9393	1:100

TH, tyrosine hydroxylase.

Supplementary Table S2. List of Commercially Available TaqMan Gene Expression Assays Used for qRT-PCR

Gene name	*Gene expression assay ID*
FOXA2	Hs00232764_m1
NR4A2	Hs00428691_m1
EN1	Hs00154977_m1
OTX2	Hs00222238_m1
CD47	Hs00179953_m1
LMX1B	Hs00158750_m1
DDC	Hs01105048_m1
KCNJ6	Hs01040524_m1
SLC6A3	Hs00997374_m1
MAP2	AX-007299-00-0200
GFAP	AX-011667-00-0200
TUBB3	AX-020099-00-0200
TH	AX-009041-00-0200
Nestin	AX-031117-00-0200
HTR2A	AX-005638-00-0200
SLC17A7	AX-007417-00-0200
CNPase	AX-018646-00-0200
MBP	AX-019663-00-0200
PAX6	AX-011098-00-0200
GAPDH	AX-004253-00-0200

Supplementary Table S3. qRT-PCR Gene Expression Data Relative to Gene Expression in DOPA2 Cells

	ΔΔCT		*ΔΔCT*			*Fold change($2^{-\Delta\Delta CT}$)*	
Gene	*2D-DOPA3*	*SD*	*3D-DOPA3*	*SD*	p	*2D-DOPA3*	*3D-DOPA3*
MAP2	−2.63	0.38	−1.96	0.96	0.33	6.19	3.90
GFAP	1.51	1.01	1.78	1.71	0.83	0.35	0.29
BIIITUB	−2.34	0.29	−1.87	0.74	0.36	5.05	3.65
TH	−3.23	0.22	−4.09	1.29	0.32	9.39	16.98
Nestin	0.72	0.39	0.30	0.65	0.39	0.61	0.81
FOXA2	4.29	0.94	2.71	1.79	0.25	0.05	0.15
NR4A2	−2.07	0.45	−1.58	0.66	0.35	4.19	2.99
EN1	0.79	1.12	1.37	2.42	0.73	0.58	0.39
OTX2	1.51	0.48	0.00	1.30	0.13	0.35	1.00
CD47	−2.46	0.21	−1.87	0.47	0.12	5.49	3.67
HTR2A	2.41	0.47	0.03	0.68	0.01	0.19	0.98
SLC17A7	−4.42	0.78	−4.54	0.59	0.85	21.40	23.20
KCNJ6	−1.39	0.59	0.26	2.06	0.25	2.62	0.84
SLC6A3	0.35	1.12	−0.70	1.97	0.47	0.79	1.63
CNPase	−0.14	0.47	−0.56	0.46	0.33	1.10	1.47
Pax6	−0.13	0.39	−0.14	0.55	0.99	1.10	1.10
LMX1B	−4.74	0.40	−4.95	2.06	0.88	26.79	30.81
DDC	−3.91	0.27	−3.69	0.34	0.44	15.01	12.92
MBP	−2.53	0.32	−3.21	0.37	0.07	5.77	9.23
SOX2	−0.49	0.30	−0.71	0.46	0.52	1.41	1.64

2D, two-dimensional; 3D, three-dimensional; SD, standard deviation.

Supplementary Table S4. qRT-PCR Gene Expression Data for 3D DOPA3 Cells (Relative to Gene Expression in 2D DOPA3 Cells)

	ΔCT		*ΔCT*				
Gene	*2D-DOPA3*	*SD*	*3D-DOPA3*	*SD*	p	*ΔΔCT*	*Fold change ($2^{-\Delta\Delta CT}$)*
MAP2	6.44	0.38	7.10	0.96	0.27	0.67	0.63
GFAP	8.98	1.01	9.25	1.71	0.82	0.27	0.83
BIIITUB	4.52	0.29	4.99	0.74	0.25	0.47	0.72
TH	11.43	0.22	10.58	1.29	0.28	−0.85	1.81
Nestin	8.88	0.39	8.46	0.65	0.26	−0.42	1.34
FOXA2	15.81	0.94	14.22	1.79	0.23	−1.58	2.99
NR4A2	9.22	0.45	9.70	0.66	0.23	0.49	0.71
EN1	15.34	1.12	15.91	2.42	0.72	0.57	0.67
OTX2	8.42	0.48	6.91	1.30	0.11	−1.51	2.85
CD47	7.11	0.21	7.69	0.47	0.10	0.58	0.67
HTR2A	18.09	0.47	15.72	0.68	0.01	−2.37	5.18
SLC17A7	14.77	0.78	14.65	0.59	0.84	−0.12	1.08
KCNJ6	12.32	0.59	13.96	2.06	0.25	1.65	0.32
SLC6A3	13.61	1.12	12.56	1.97	0.47	−1.05	2.07
CNPase	10.02	0.47	9.60	0.46	0.31	−0.42	1.34
Pax6	15.47	0.39	15.47	0.55	0.99	0.00	1.00
LMX1B	14.50	0.40	14.30	2.06	0.87	−0.20	1.15
DDC	7.58	0.27	7.80	0.34	0.31	0.22	0.86
MBP	14.13	0.32	13.45	0.37	0.04	−0.68	1.60
SOX2	5.23	0.30	5.01	0.46	0.46	−0.22	1.17

Supplementary References

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