SUPPLEMENTAL FIGURES

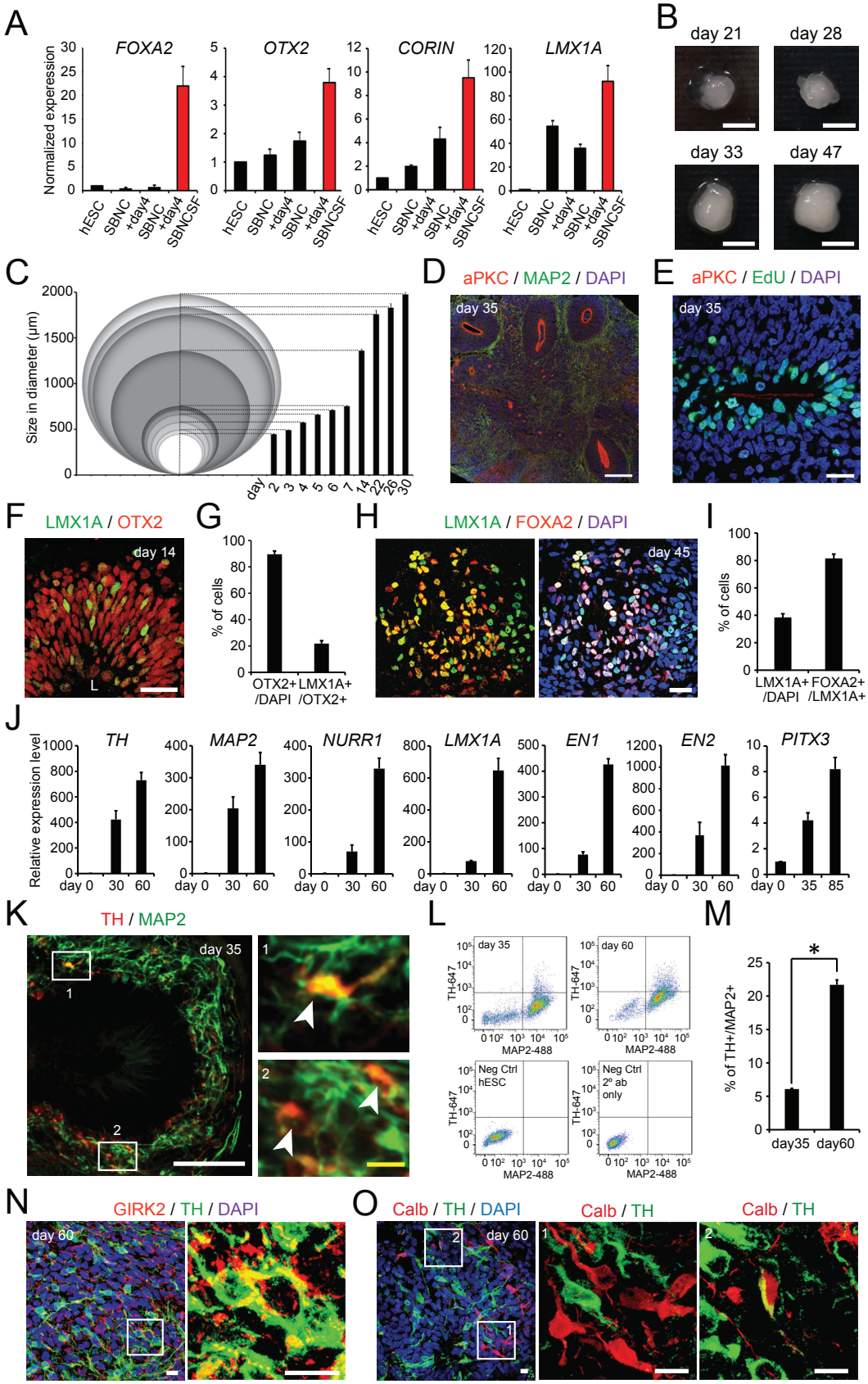

Figure S1, related to Figure 1. Characterization of hMLOs and characterization of mDA neurons in hMLOs. (A) Quantitative RT-PCR analysis of cells dissociated from either hESCs or hMLOs treated with

SBNC or SBNC+SF for *FOXA2*, *OTX2*, *CORIN*, and *LMX1A* (midbrain progenitor markers). **(B)** DIC images illustrate the typical morphology of hMLOs at each stage as indicated. Scale bar = 2mm. **(C**) Growth rates of hMLOs at each stage as indicated **(D)** Cryosection of an hMLO at day 35 stained for aPKC and MAP2. Note multiple round shaped rosettes in the hMLO. Scale bar = 200μm. **(E)** Cryosection of an hMLO at day 35 stained for aPKC and EdU, showing that proliferating EdU-positive cells were located at the apical surface of a neuroepithelium. Scale bar = 20μm. **(F)** Cryosection of a day 14 hMLO labeled for LMX1A and OTX2. The quantifications are shown in **(G)**; mean ± s.e.m., *n*=3. Scale bars = 50μm. **(H)** Cryosection of an hMLO at day 45 that was stained for LMX1A and FOXA2. Scale bars = 20μm. The quantifications are shown in **(I)**; mean ± s.e.m., *n*=3. **(J)** Quantitative RT-PCR analysis of cells dissociated from hMLOs for *TH*, *MAP2*, *NURR1*, *LMX1A*, *EN1*, *EN2*, and *PITX3*. **(K)** TH-positive neurons are located in the MZ at day 35. White scale bar = 100μm. Yellow scale bar = 10μm. Arrowheads indicate TH and MAP2 double-positive cells. **(L and M)** Flow cytometric analysis of cells dissociated from hMLOs at day 35 and day 60 to quantify the percentage of mDA neurons (TH+/MAP2+), revealing a significant increase at day 60; mean ± s.e.m., *n*=3. Bottom left: flow cytometry analysis of hESC cells stained for both primary and secondary antibodies. Bottom right: flow cytometry analysis of hMLO-derived cells stained only with secondary antibodies. Threshold gate is based on the both negative control that used to quantify the percentage of cells expressing TH and MAP2. **(N)** GIRK2 and TH double-positive neurons within the MZ of a day 60 hMLO including a higher-magnification view to better illustrate the colocalization. Scale bar = 10μm. **(O)** Cryosection of an hMLO at day 60 immunostained for TH and Calbindin (Calb, a marker for A10 subtype of DA neurons), including a higher-magnification view to illustrate cells that were positive for either TH or Calb (1) and cells were double positive for both TH and Calb (2). Scale bar = 10μm.

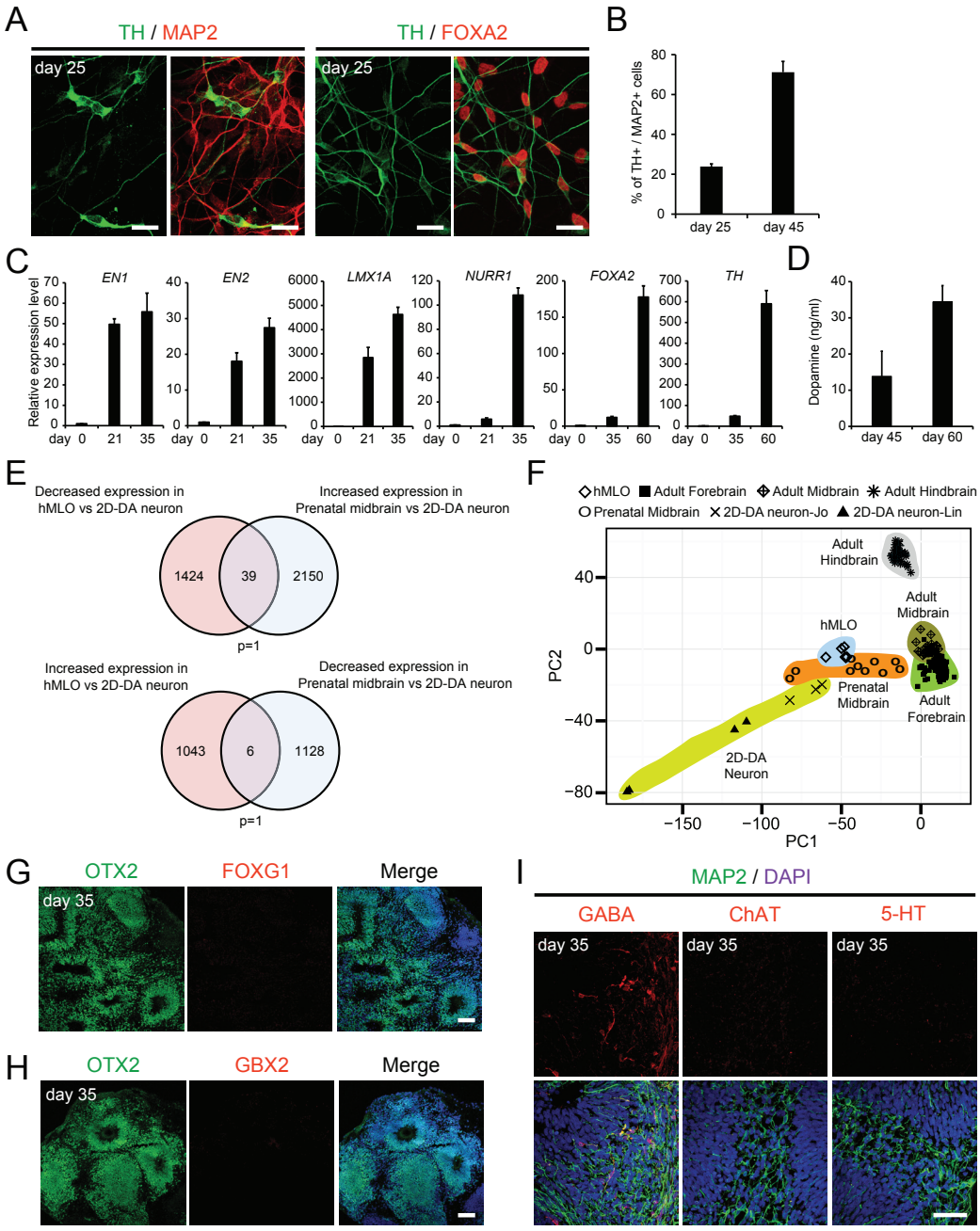

Figure S2, related to Figure 2. (A-D) Characterization of 2D-DA neuron, (E-F) Transcriptional Characterization of hMLOs, (G-I) Regional and cellular identity of hMLOs. (A) Immunostaining of day 25 2D-DA neurons for DA neuron marker TH and FOXA2. **(B)** Quantifications of TH+/MAP2+ cells at day 25 and 45. (B); mean ± s.e.m., n=5. Scale bar = 20µm. **(C)** Quantitative RT-PCR analysis of 2D-DA neurons for, EN1, EN2, LMX1A, NURR1, FOXA2, and TH. **(D)** Dopamine content measured in 2D-DA neurons by HPLC (n=3). **(E)** Venn Diagram indicating the overlap of upregulated and downregulated genes in hMLOs and prenatal midbrain, compared to 2D-grown human DA neurons. (Significance was estimated using Fisher's exact test) (related to Figure 2C). **(F)** Principal component analysis (PCA) of the RNA-Seq data from adult forebrain, adult hindbrain, adult midbrain, prenatal midbrain, hMLOs, and 2D-DA neurons (2D-DA neuron-1: our RNA-seq data; 2D-DA neuron-2: RNA-seq data based on (Lin et al., 2016). The PCA was calculated using the top 1,000 genes with the highest variance in normalised read counts across all samples (regularized log transformation). Histone genes were removed for this analysis. **(G)** Cryosection of an hMLO at day 35 that was stained for OTX2 (midbrain marker) and FOXG1 (forebrain marker). Scale bar = 100µm. **(H)** Cryosection of an hMLO at day 35 stained for OTX2 and GBX2 (hindbrain marker). **(I)** Cryosection of an hMLO at day 35 that was stained for GABA (GABAergic neuronal marker), ChAT (cholinergic neuronal marker), and 5-HT (serotonergic neuronal marker). Scale bar = 50µm.

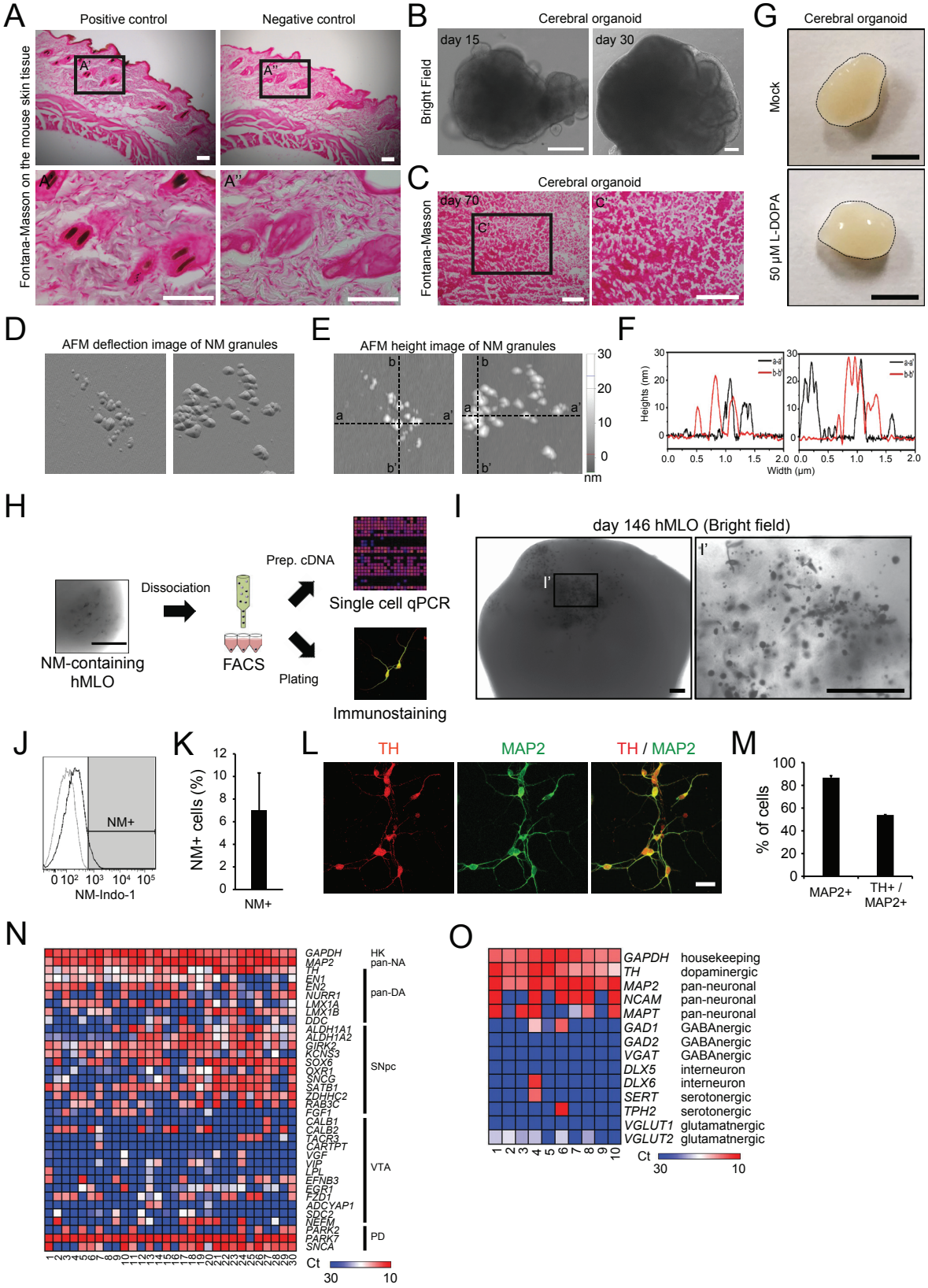

Figure S3, related to Figure 3. Characterization of NM granules isolated in the hMLOs and transcriptional characterization of NM-containing cells. (A) Fontana-Masson staining on cryosections of an adult mouse skin tissue, which exhibited melanin deposits. Left columns: positive control. A' is an enlarged view of a region in the upper left panel. Right columns: negative control by removing one component (ammonical silver nitrate solution) of Fontana-Masson staining. A'' is an enlarged view of a region in the upper right panel.

Scale bar = 100μm. **(B)** Representative DIC images of human cerebral organoid at day 15 and 30. Scale bar = 200μm. **(C)** Cryosection of a human cerebral organoid (hCO) at day 70, stained with Fontana-Masson staining. Note the lack of NM-like granules in the hCO. Scale bar = 100μm. **(D and E)** Deflection and height images of NM granules, isolated from the day 122 hMLOs. Fixed NM granules were imaged using atomic force microscopy (AFM). **(F)** The height profiles of AFM along the a-a' axis (black) and b-b' axis (red). Actual size of all processed AFM images was 2 x 2 μm. **(G)** The hCOs were treated with vehicle (Mock) and L-DOPA (50μM) for 10 days, and hCOs did not show any NM-like granules and colored deposits (related to Figure 3G). Scale bar = 2mm. **(H)** Schematic diagrams illustrating the experiment to investigate NM-containing cells. **(I)** The representative images of a day 146 hMLO, which was utilized for sorting of NM-containing cells. Scale bar =200μm. **(J and K)** Flow cytometric analysis of cells dissociated from hMLOs at day120-150 to quantify the percentage of NM-containing cells. **(L)** Immunostaining of sorted NM-containing cells with antibodies against MAP2 and TH. Scale bar = 50μm. **(M)** Quantification of MAP2+ and TH+/MAP2+ cells after NM FACS sorting using qPCR; mean ± s.e.m., *n*=3. **(N and O)** Single-cell gene expression profiling to identify NM-containing mDA neurons from the day 120-150 hMLOs using a microfluidic dynamic array. Expression levels (shown as Ct values) are color-coded at the bottom (HK, housekeeping genes; pan-NA, pan-neuronal genes; pan-DA, pan-dopaminergic neuronal genes; SNpc, *substantia nigra pars compacta*-related genes; VTA, ventral tegmental area-related genes; PD, Parkinson's disease-related genes).

SUPPLEMENTAL TABLES

Table S1. Differential expression analysis between 2D-DA neurons and hMLOs. The table contains gene annotation, fold change, adjusted p-values and normalized expression estimates, related to Figure 2A.

Table S2. Differentially expressed genes in the hMLOs and prenatal midbrain compared to 2D-DA neurons, related to Figure 2D.

Table S3. Quantitative analysis of NM granules. The data was calculated by imaging process program, related to Figure 3E, F, and S3D-F (XEI, Parksystem Corp., Suwon, Korea).

	Height (nm)	**Diameter (nm)**	**Volume (μm^3)**	**Height (nm)**	**Diameter (nm)**	**Volume (μm^3)**
	hMLO day122 NM (Figure S3D, E, and F, Left)			hMLO day122 NM (Figure S3D, E, and F, Right)		
Average	16.96	125.06	6.86E-05	22.47	156.79	2.73E-04
STDV	4.57	62.99	7.54E-05	5.79	111.61	4.49E-04
	Human postmortem *substantia nigra pars compacta* NM			Human postmortem *substantia nigra pars compacta* NM		
Average	15.56	119.5	7.65E-05	14.41	151.81	9.78E-05
STDV	4.1	74.5	5.04E-05	3.52	110.16	3.67E-05

Table S4. Summary of major electrophysiological parameters of neurons recorded from the hMLOs, related to Figure 4.

Neurons number	Cm (pF)	RM (MOhm)	Ra (MOhm)	RMP (mV)	AP-threshold (mV)	Spike AHP (mV)	Half AP width (ms)
#1	16	1000	6	-48	-35	12.37	1.14
#2	18	1000	8	-58	-30	9.33	2.32
#3	9	2500	12	-45	-	-	-
#4	12	2000	11	-50	-	-	-
#5	24	1400	16	-38	-27	18.31	1.68
#6	13	2000	12	-42	-	-	-
#7	34	1400	13	-46	-	-	-
#8	37	1000	9	-42	-	-	-
#9	10	1600	10	-48	-48	4.54	2.1
#10	25	1200	12	-52	-37	5.91	1.84
#11	29	1200	9	-55	-	-	-
#12	19	2100	13	-48	-35	17.4	1.62
#13	13	2000	11	-39	-28	7.46	2.52
#14	13	1200	12	-43	-24	5.17	2.75
#15	29	1000	12	-60	-34	10.18	1.34
#16	19	1800	10	-55	-35	6.05	3.15
#17	13	1000	10	-45	-29	8.75	1.82
#18	13	1200	13	-48	-28	11.6	2.33
#19	18	1000	8	-58	-33	9.98	1.42
#20	30	1200	12	-53	-40	5.95	1.14
#21	22	1600	6	-67	-40	12.43	1.01
#22	44	800	13	-60	-44	12.86	1
#23	35	900	11	-53	-36	9.03	1.84
#24	28	1000	14	-48	-	-	-
#25	17	700	7	-57	-	-	-
#26	36	1100	10	-40	-28	11.33	2.53
Average (at day 33-50)	20.50±2.70	1533±145.8	10.92±0.77	-47.67±1.64	-35.33±2.95	11.31±2.353	1.78±0.17
Average (at day 65-84)	23.57±2.71	1179±100.1	10.64±0.63	-51.86±2.22	-33.25±1.75	9.23±0.76	1.90±0.21

Table S5. Information for each human prenatal midbrain samples that was performed RNA-seq, related to Figure 2.

Name of sample	Gestational Age (WW/D)	Sex	Race
Prenatal Midbrain_1	17 / 4	Male	caucasian
Prenatal Midbrain_2	17 / 5	Male	caucasian
Prenatal Midbrain_3	17 / 3	Male	caucasian
Prenatal Midbrain_4	17 / 0	Male	caucasian
Prenatal Midbrain_5	17 / 0	Male	caucasian
Prenatal Midbrain_6	16 / 0	Female	caucasian
Prenatal Midbrain_7	15 / 0	Female	caucasian
Prenatal Midbrain_8	13 / 2	-	caucasian
Prenatal Midbrain_9	13 / 2	Female	caucasian
Prenatal Midbrain_10	13 / 0	-	caucasian

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Culture of human pluripotent stem cells (hPSCs)

The hESC lines H1 (WA01, passage 30) and H9 (WA09, passage 25) were used. The hPSCs were maintained under feeder-free conditions over Matrigel-coated 6-well plates (BD Biosciences) in mTeSR media (Stemcell Technologies) at 37°C in a humidified CO_2 incubator. The media was changed daily, and the hPSC cultures were split every 5 days using 1 mg/ml Dispase (Stemcell Technologies). All hPSC lines were confirmed negative for mycoplasma contamination and exhibited normal karyotypes.

Generation of human midbrain-like organoid

hPSC lines before passage 40 were used to generate hMLOs. The hPSCs were dissociated from intact colonies to single cells with TrypLE™ Express (Life Technologies), and 10,000 cells were plated in each well of low-cell-adhesion 96-well culture plates with V-bottomed conical wells (Sumitomo Bakelite) to form uniform EBs. The wells contained neuronal induction medium containing DMEM/F12:Neurobasal (1:1), 1:100 N2 supplement (Invitrogen), 1:50 B27 without vitamin A (Invitrogen), 1% GlutaMAX (Invitrogen), 1% minimum essential media-nonessential amino acid (Invitrogen), and 0.1% β-mercaptoethanol (Invitrogen) supplemented with 1μg/ml heparin (Sigma-Aldrich), 10μM SB431542 (Stemgent), 200 ng/ml Noggin (Prospec), 0.8μM CHIR99021 (Reagents Direct), and 10μM ROCK inhibitor Y27632 (Calbiochem). The ROCK inhibitor was added for the first 48 h, and the neuronal induction medium was changed on day 2. On day 4, hMLOs were cultured with the addition of 100 ng/ml SHH-C25II (R&D Systems) and 100 ng/ml FGF8 (R&D Systems) for midbrain patterning. After 3 days, the hMLOs started to extrude neuroectodermal buds. At this point, the media was completely removed, and 30μl of reduced growth factor Matrigel was immediately added to each well using a pipettor equipped with a pre-chilled 200μl pipette tip. The Matrigel-embedded midbrain organoid was placed into a 37°C incubator for 30 min to allow the Matrigel to solidify and was grown in tissue growth induction medium containing Neurobasal medium, 1:100 N2 supplement (Invitrogen), 1:50 B27 without vitamin A (Invitrogen), 1% GlutaMAX (Invitrogen), 1% minimum essential media-nonessential amino acid (Invitrogen), and 0.1% β-mercaptoethanol (Invitrogen) supplemented with 2.5 μg/ml insulin (Sigma-Aldrich), 200 ng/ml laminin (Sigma-Aldrich), 100 ng/ml SHH-C25II (R&D Systems), and 100 ng/ml FGF8 (R&D Systems) for 24 h. Once the hMLOs were embedded in Matrigel, a more expanded neuroepithelium began to form in the organoids. To promote growth and differentiation, the hMLOs were transferred into ultra-low-attachment 6-well-plates (Costar) by pipetting using a cut 1000 μl pipette tip. The plates contained the final differentiation media, which consisted of Neurobasal medium, 1:100 N2 supplement (Invitrogen), 1:50 B27 without vitamin A (Invitrogen), 1% GlutaMAX (Invitrogen), 1% minimum essential media-nonessential amino acid (Invitrogen), 0.1% β-mercaptoethanol (Invitrogen), 10ng/ml BDNF (Peprotech), 10 ng/ml GDNF (Peprotech), 100 μM ascorbic acid (Sigma-Aldrich), and 125 μM db-cAMP (Sigma-Aldrich). The hMLOs were cultured using an orbital shaker to enhance nutrients and oxygen exchange (Lancaster and Knoblich, 2014). Antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) were included in the culture media to prevent potential bacterial contamination over long-term culture. The medium was replaced every 3 days.

Immunohistochemistry

The hMLOs were fixed in 4% paraformaldehyde (PFA) overnight, incubated in a 30% sucrose solution in PBS at 4°C overnight, and subsequently embedded in O.C.T. compound (Sakura Finetek) for cryosectioning. Frozen hMLOs were cryosectioned at a thickness of 16μm. For immunohistochemistry, hMLO cryosections were blocked with 3% BSA and 0.5% Triton X-100 in PBS for 1 h at room temperature. The sections were incubated with primary antibodies diluted overnight and secondary antibodies for 1 h at room temperature. All sections were counterstained with DAPI (Sigma-Aldrich) and mounted with Aqua-mount (Thermo) or kept in PBS, protected from light. Images were taken on an LSM 710 confocal microscope or an Observer Z.1 inverted microscope (Zeiss).

RNA extraction, reverse transcription, real-time RT-PCR, and RNA-seq preparation

Total RNAs were isolated from either hMLOs, 2D-DA neurons, or undifferentiated hESCs using TRIzol Reagent (Invitrogen). DNA contaminants were digested with DNASE I (Ambion) and 500 ng of RNA was reverse-transcribed using the SuperScript II kit (Invitrogen) to produce the cDNA. Quantitative RT-PCR was performed using ViiA 7 Real-Time PCR system (Applied Biosystem). ΔΔCt method was applied to normalize expression levels of each gene to that of GAPDH. Primers used were priorly tested to ensure specific melting curves and additionally validated by using human cDNA library, prepared from human neuronal samples (ScienCell). For RNA-seq library preparation of hMLOs and 2D-DA neurons, the total RNA was further purified by column (Puelink RNA Mini kit, Ambion), and was prepared using 4μg of total RNA according to manufacturer's protocol (TruSeq RNA Sample Preparation Kit v2, Illumina). Samples were

multiplexed and sequenced single read 150bp paired end (HiSeq 2000, Illumina).

Human brain dissection

The ventral half of the midbrain was dissected from second trimester prenatal brainstem under visual inspection, using a scalpel and hand-held dental drill (Table S5). Cases were donated with informed consent of the mother, and were selected on the basis of the absence of any congenital anatomical abnormality on macroscopic brain inspection, and the absence of any major genetic defect noted on prenatal testing. The IRB approval is from the Western IRB, a private and fully accredited IRB that is used by many independent institutions in the USA: address 1019 39th Avenue SE Suite 120, Puyallup, WA 98374-2115. Study number: 1126332. WIRB protocol number: 20111080. Approval expires: 7/18/2016.

Post-mortem brain: RNA extraction and sequencing

Details of post-mortem RNA Extraction and Sequencing, and RNA-seq data processing were previously described (Jaffe et al., 2015). Post-mortem tissue homogenates of Ventral Midbrain were obtained from prenatal brains. Following RNA extraction, RNA quality was measured using high-resolution capillary electrophoresis on an Agilent Technologies Bioanalyzer 2100, and RNA integrity numbers (RINs) were acquired for all the 10 samples.

Ribosomal RNA depeletion (RiboZero) with strand-specific library preparation and Q/C

RNA-seq libraries were constructed using Illumina TruSeq Stranded Total RNA Ribo-Zero sample Prep Kit following the manufacturer's protocol. The ribosome RNAs were removed using Ribo-zero beads from ~ 800 ng DNAse treated total RNA. Following purification, the total RNA without Ribosome RNA was fragmented into small pieces using divalent cations under elevated temperature (94 degree) for 2 minutes. Under this condition, the range of the fragments length is from 130-290 bp with a median length of 185 bp. Reverse transcriptase and random primers were used to copy the cleaved RNA fragments into first strand cDNA. The second strand cDNA was synthesized using DNA Polymerase I and RNaseH, dUTP in place of dTTP. These cDNA fragments then went through an end repair process using T4 DNA polymerase, T4 PNK and Klenow DNA polymerase, and the addition of a single 'A' base using Klenow exo (3' to 5' exo minus), then ligation of the Illumina PE adapters using T4 DNA Ligase. An index (96 unique dual-index pairs) was inserted into Illumina adapters so that multiple samples can be sequenced in one lane of 8-lane flow cell if necessary. These products were then purified and enriched with 15 cycles of PCR to create the final cDNA library for high through put DNA sequencing using the H 100 base-pair paired-end reads and the Illumina HiSeq 3000, targeting approximately 100M reads per sample. The concentration of RNA libraries was measured by Qubit (Invitrogen, CA). The quality of RNA-seq library was measured by LabChipGX (Caliper, MA) using HT DNA 1K/12K/HiSens Labchip.

Genotype-Tissue Expression (GTEx) acknowledgment

The GTEx Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health. Additional funds were provided by the NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. Donors were enrolled at Biospecimen Source Sites funded by NCI\SAIC-Frederick, Inc. (SAIC-F) subcontracts to the National Disease Research Interchange (10XS170), Roswell Park Cancer Institute (10XS171), and Science Care, Inc. (X10S172). The Laboratory, Data Analysis, and Coordinating Center (LDACC) was funded through a contract (HHSN268201000029C) to The Broad Institute, Inc. Biorepository operations were funded through an SAIC-F subcontract to Van Andel Institute (10ST1035). Additional data repository and project management were provided by SAIC-F (HHSN261200800001E). The Brain Bank was supported by a supplements to University of Miami grants DA006227 & DA033684 and to contract N01MH000028. Statistical Methods development grants were made to the University of Geneva (MH090941 & MH101814), the University of Chicago (MH090951, MH090937, MH101820, MH101825), the University of North Carolina - Chapel Hill (MH090936 & MH101819), Harvard University (MH090948), Stanford University (MH101782), Washington University St Louis (MH101810), and the University of Pennsylvania (MH101822). The data used for the analyses described in this manuscript were obtained from dbGaP accession number phs000424.v6.p1 on 04/09/2015.D.K. and H.S.K were supported by grants from the NIH (NS082205 and NS38377) and the Maryland Stem Cell Research Foundation (RFA-MD-13-2). S.O.K., J.H.P., and N.J.C.were supported by the National Research Foundation in Singapore (NRF-NRFF2011-01).

Bioinformatics analysis

RNA-Seq data were mapped against the human genome version hg19 with TopHat2-2.0.12 (Kim et al., 2013) using the GENCODE Release 19 version of gene annotations. R-3.1.2 (Team, 2014) and Bioconductor 3.0 (Gentleman et al., 2004) were used for the RNA-Seq analysis. Reads were counted using the R package

GenomicAlignments (Lawrence et al., 2013) (mode='Union', inter.feature=FALSE), only primary read alignments were retained. Rlog transformed values of the counts and differential expression was calculated using DESeq2 (Love et al., 2014). We used an adjusted p-value threshold of 0.0005, and a log fold change threshold of 2 (-2) for up- and downregulated genes respectively. Coverage plots (Figure 2E and F) and PCA (Figure S2F) were created using ggplot2_1.0.0 (Wickham, 2009). Adult brain RNA-Seq data from the GTEx project (Consortium, 2015) were used as a comparison. In order to plot gene expression in heatmaps, genes were sorted by fold change, and the distribution of expression estimates for each gene were standardized for visualization by subtracting the mean and dividing by standard deviation.

Flow cytometry

For intracellular staining, cells were fixed and permeabilized with transcription factor staining buffer set (eBioscience). Briefly, cells were firstly dissociated with TrypLE™ Express (Gibco), spun down and cell pellets were resuspended in 1ml 1x fixation / permeabilization buffer in the dark at 4°C for 30 min. Without washing to minimize loss of cell, 2ml of 1x permeabilization buffer was added to cell suspension and samples were spun down. The supernatant was decanted and samples were stained with antibodies in 100µl permeabilization buffer at room temperature for 1 h. Finally, 300 µl of staining buffer was added to cell suspension until analysis. Cells were filtered through BD Falcon 12x75mm tube with cell strainer (BD Biosciences). Flow cytometry analysis was performed using a BD LSRFortressa™ X-20 (BD Biosciences), and all data were represented by FlowJo software. Negative threshold gates were defined with two negative control samples (dissociated hMLO cells stained with secondary antibody alone and hESC stained with both primary and secondary antibodies).

Dopaminergic neuron induction (2D method)

DA neuron differentiation was performed follow floor-plate based neural induction protocol (Kriks et al., 2011). The hESCs were plated at 35 x 10^3 cells per cm^2 and grown on matrigel in Basal medium 1 containing DMEM, 15% KSR, 2mM L-Glutamine and 10µM β-mercaptoethanol, with addition of 100nM LDN193189 (Stemgent) and 10µM SB431542 for 24 h. On day 1, medium was changed to Basal Medium 1 with addition of 100nM LDN193189, 10µM SB431542, 100ng/ml SHH-C25II, 2µM Purmorphamine (Stemgent) and 100ng/ml FGF8 for 2 days. On day 3, medium was changed with addition of 3µM CHIR99021. By day 5, medium was gradually shifted to N2 medium containing DMEM, N2 supplement, 2mM L-Glutamine and 10µM β-mercaptoethanol (Basal Medium 2) for 2 days interval as described previously (Chambers et al., 2009). On day 11 onwards, Basal medium 3 containing Neurobasal/B27/L-Glutamine (Invitrogen) was changed supplemented with 3µM CHIR99021 for 2 days. After which, Basal medium 3 supplemented with 10µM DAPT (Sigma-Aldrich), 0.2mM Ascorbic acid, 20ng/ml BDNF, 20ng/ml GDNF, 0.2mM db-cAMP and 1ng/ml TGF-β3 (Invitrogen) was used until day 20, media were changed every other day. On day 20, cells were dissociated using 0.05% Trypsin-EDTA and replated under high cell density (300 - 400 x 10^3 cells per cm^2) on dishes pre-coated with 0.1mg/ml poly-lysine and 1µg/ml Laminin. Cells were grown in Neurobasal/B27 medium containing 10µM DAPT, 0.2mM Ascorbic acid, 20ng/ml BDNF, 20ng/ml GDNF, 0.2mM db-cAMP and 1ng/ml TGF-β3, and changed fresh medium every other day until experimental analysis as indicated.

Generation of cerebral organoid

Cerebral organoids were generated using the same protocol as Lancaster et al (Lancaster and Knoblich, 2014; Lancaster et al., 2013). Briefly, hESCs were dissociated with TrypLE™ Express, and 10,000 cells were plated in each well of low-cells-adhesion 96-well culture plate with V-bottomed conical wells to form EBs in DMEM/F12, and 20% Knockout serum replacement (Gibco), 1% GlutaMax (Invitrogen), 1% NEAA (Invitrogen), 0.1% β-mercaptoethanol (Invitrogen), 4ng/ml of bFGF (Invitrogen), and 20µM ROCK inhibitor Y27632 (Calbiochem). At day6, EBs were transferred to low adhesion 24-well plate in neural induction media containing DMEM/F12, 1:100 N2 supplement (Invitrogen), 1% GlutaMax (Invitrogen), 1% NEAA (Invitrogen), and 1µg/ml Heparin (Sigma-Aldrich). The EBs began to form neuroepithelium, and transfer to embed in Matrigel (BD Bioscience) at day11 on a sheet of Parafilm with small dimples. The Matrigel droplets were solidified at 37°C and grown in differentiation media containing DMEM/F12:Neurobasal (1:1), 1:100 N2 supplement (Invitrogen), 1:50 B27 without vitamin A (Invitrogen), 1% GlutaMAX (Invitrogen), 1% NEAA (Invitrogen), 0.1% β-mercaptoethanol (Invitrogen), 2.5 µg/ml Insulin (Sigma-Aldrich), and 100 U/ml penicillin G, 100 µg/ml streptomycin (Gibco-BRL) to allow stationary growth. At day15, the droplets were transferred onto ultra-low attachment 6-well plate (Costar) in final media containing B27 supplement with vitamin A (Invitrogen) in differentiation media. The cerebral organoids were cultured on orbital shaker. The media was replaced every 3 days.

Neuromelanin staining (Fontana-Masson staining)

The hMLOs were fixed in 4% PFA overnight, washed in PBS, embedded in paraffin, and sectioned at a thickness of 4µm. Next, the hMLO sections were deparaffinized, hydrated with distilled water, and stained with a Fontana-Masson staining kit (Abcam) according to the manufacturer's protocol (Carriel et al., 2011). Briefly, the sections were placed in the ammoniacal silver solution and incubated in a 58 – 60°C water bath for 30 – 60 min until they become yellowish/brown in color. The sections were then washed with distilled water and incubated in the gold chloride solution (0.2%) for 30 sec. Next, the sections were incubated in the gold chloride solution (0.2%) for 30 sec and subsequently incubated with the sodium thiosulfate solution (5%) for 2 min. Finally, the sections were incubated in the Nuclear Fast Red solution for 5 min for staining and mounting. Images were taken on an Observer Z.1 uplight microscope (Zeiss).

Neuromelanin isolation and quantification

The hMLOs that were positive for NM granules were homogenized in a glass tube with 1.5 ml of pH 7.4 phosphate buffer (50mM), followed by the centrifugation at 12,000 g for 30 min. The resulting pellet was washed with pH 7.4 phosphate buffer twice, and then was resuspended with 1.5ml of Tris buffer (50mM, pH 7.4), containing sodium dodecyl sulfate (5mg/ml) and 0.2 mg/ml proteinase K (Fermentas) using a shaker, for 3 h at 37°C. Resuspended NM granules were subsequently centrifuged at 12,000g for 30 min, and then washed twice with NaCl (9 mg/ml), and twice with distilled water. For the quantification of NM granules, NM granules were solubilized in 1ml of 1 M NaOH at 80°C for 1h to measure NM by spectrophotometer (350 nm). The solution is centrifuged and the supernatant was collected. For the imaging of NM structure by AFM and SEM, the pellet was washed with 1.5 ml of methanol and 1ml of hexane. Lastly, it was dried by lyophilization (Bush et al., 2006; Zecca et al., 2002).

Atomic force microscopy (AFM)

In this measurement, commercial AFM system (NX-Bio, Park Systems Corp., Korea) was used. The Z scanner mounted on the AFM head makes it possible for the probe to maintain constant feedback conditions (force or distance) as it is moved over a sample surface. It is also combined with commercial inverted optical microscopy (Ti eclipse, Nikon, Japan) which enables easily detect the sample and AFM probe position. All images were performed using AFM contact mode in air condition at room temperature. The scanning area was 2 × 2 μm^2, the resolution was 256 × 256 pixels. The height and deflection images were collected simultaneously with 0.8 – 1.2 Hz of scan rate. To obtain a reliable image, we used very soft commercial Si cantilevers (CSG-01, NT-MDT, Russia) which was cleaned with an UV/ozone cleaner for 15 min. Spring constants (0.06 N/m) and resonant frequency (12 kHz) of the probes were evaluated by measurement of thermal fluctuations of the probes. For AFM imaging, NM samples were prepared by pipette dropping of NM solution (5 µl) onto cleaned and cleaved mica surface. The NM samples on mica surface were stored and dried in the dark condition until NM is fully dry.

Scanning Electron Microscopy (SEM)

The procedures used for AFM sample preparation as discussed above were implemented for the NM samples prior to SEM analysis. The pre-dried samples were then sputter-coated with a 10 nm thick layer of gold for 50 seconds at 20 mA using Auto Fine Coater (JEOL JFC-1600, Japan), and SEM images were processed with Field Emission Scanning Electron Microscopy (JEOL 6340F, Japan) equipped with a cold cathode emitter set at an acceleration voltage of 5.0 kV.

Single-cell gene expression analysis

The 120 to 150 days hMLOs were dissociated to single cells by TrypLE™ Express and the cells were filtered through BD Falcon 12x75mm tube with cell strainer. To collect NM contained cells, cells were sorted directly with the BD FACSAria II (BD Bioscience) into 96-well PCR plates loaded with 5 µl of the reverse transcription-specific target amplification solution (Life Technologies) based on detection using excitation laser 355nm and filter 530/30 (Nighswander-Rempel et al., 2005). For positive control, 2D differentiated DA neurons were suspended and put directly with same FACS instrument in 96-well PCR plates. The reverse transcription (RT)-specific target amplification was carried out in thermal cycler with 20-cycle preamplification and unused primers were digested away by exonuclease 1 (New England BioLabs) treatment, and then samples were diluted 3-fold prior to the analysis. The preamplified products in 96-well PCR plates were validated expression of *GAPDH, MAP2, and TH* by qRT-PCR, and we removed cells to be shown weak or no expression of those genes. For characterization of mDA neuron in hMLO, we analyzed cells except those cells. These samples were analyzed with 2X SsoFast EvaGreen Supermix with Low ROX (Bio-Rad) and individual qPCR primers in 96x96 Dynamic Arrays on a Biomark System (Fluidigm), following manufacture's instruction. Ct values were calculated using the BioMark Real-Time PCR Analysis software (Fluidigm). Results were represented as a

heatmap by color-coded Ct values in Figure S3N and O. Each primer pair was designed and validated to ensure specific melting curves and additionally evaluated by using commercial reference sample [human cDNA library prepared from human neurons (ScienCell)].

Electrophysiology

Whole cell patch clamp recording techniques were used in this study. Each hMLO was placed in a chamber and submerged beneath continuously perfused artificial CSF (aCSF) containing (in mM): 119 NaCl, 2.5 KCl, 11 glucose, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 $CaCl_2$ and 1.3 $MgCl_2$ saturated with 95% O_2 and 5% CO_2 at 30-32 °C. Patch pipettes (2-4MΩ) were filled with an intracellular solution containing (in mM): 135 potassium methanesulfonate, 10 KCl, 10 HEPES, 1 EGTA, and 2 Na_2ATP. The osmolality was 290 mOsm, and the pH was adjusted to 7.2-7.4 with KOH.

The whole cell recordings were performed using IR-DIC visualization techniques with an Olympus BX51WI upright microscope equipped with a 60x water-immersion lens. Signals were recorded using a MultiClamp 700B amplifier, filtered at 3 kHz using a Bessel filter, and digitized at 10kHz with a Digidata 1322A analog-to-digital (A/D) board (Molecular Devices, Sunnyvale, CA). Biocytin (0.3%; Sigma-Aldrich) was freshly dissolved in the pipette solution before recordings. Resting membrane potential was estimated in current-clamp mode immediately after breaking the membrane and establishing whole-cell configuration. To measure Na^+ currents and K^+ currents, voltage steps (500 msec duration) were applied from a holding potential of -60 mV to a range of test potentials between -50 and +90mV (in 10 mV increments) in voltage-clamp mode. sEPSCs were collected at a holding potential of – 70 mV, while evoked excitatory postsynaptic currents (eEPSCs) were induced by tungsten wire electrodes placed within the hMLO ~ 300μm away from the recorded neuron. Spontaneous action potentials were measured in current-clamp mode (0 pA). In some experiments, quinpirole (1 μM, sigma-Aldrich) was applied to suppress the spontaneous action potential. To test the cells' excitability, a series of current pulses (500 msec) of increasing amplitude (in 5pA steps) were injected to obtain the current-firing frequency relationship. The rebound action potentials were induced by brief hyperpolarizing current injections (-20 pA).

High-performance Liquid Chromatography (HPLC)

Single hMLO, human cerebral organoid (hCO), or 1 well of 2D-DA neurons in 6 well dish was homogenized in 100μl of 0.5N perchloric acid, centrifuged, and filtrated through 0.1 mm filters (Millipore). Only resulting supernatants were loaded into an HPLC system (Thermo Scientific). The mobile phase for dopamine is run 12.5% acetonitrile buffer (pH 3.0, 90 mM sodium phosphate monobasic dehydrate, 50mM citric acid, 2.1 mM 1-octanesulfonate monohydrate, and 0.1 mM EDTA). We used Dionex Coulochem III Electrochemical detector to determine dopamine levels via a customized program with a sensitivity range of 2-10nA. The results were then analyzed through the normalization of cell counts and conversion of peak reads to numerical expression.

Statistical analysis

All experiments were conducted at least in triplicate, and the results are expressed as the mean ± standard error. Statistical analyses were performed using Two-tailed Student's *t*-test. A $p<0.05$ was considered statistically significant.

Antibodies

Antibody	Host species	Company	Cat. No	Dilution
Ki67	Mouse	BD Biosciences	550609	1:200
MAP2	Chicken	ABcam	ab5392	1:5000
OTX2	Goat	Neuromics	GT15095	1:1000
OTX2	Rabbit	ABcam	Ab21990	1:1000
MASH1	Mouse	BD Biosciences	556604	1:200
aPKC	Mouse	Santa Cruz	Sc-17781	1:100
FOXA2	Rabbit	ABcam	ab108422	1:250
LMX1A	Goat	Santa Cruz	Sc-54273	1:100
NURR1	Rabbit	Santa Cruz	sc-991	1:200
TH	Rabbit	PelFreez	P40101-0	1:1000
TH	Mouse	ImmunoStar	22941	1:1000
DAT	Rat	Millipore	MAB369	1:2000
GIRK2	Rabbit	Alomone	APC-006	1:500
CALBINDIN	Rabbit	Swant	D-28k	1:20000
FOXG1	Rabbit	ABcam	ab18259	1:2000
GBX2	Goat	Santa Cruz	Sc-22230	1:100
GABA	Rabbit	Sigma	A2052	1:1000
ChAT	Goat	Millipore	AB144P	1:100
5-HT	Rabbit	Immunostar	20080	1:1000

Primers

Gene	Entrez ID	Forward	Reverse
GAPDH	2597	CAAGATCATCAGCAATGCCTCCTG	GCCTGCTTCACCACCTTCTTGA
OCT4	5460	GTGGAGGAAGCTGACAACAA	ATTCTCCAGGTTGCCTCTCA
NANOG	79923	TTTGTGGGCCTGAAGAAAACT	AGGGCTGTCCTGAATAAGCAG
PAX6	5080	TCCACCCGGCAGAAGATTGTA	TGTCTCGGATTTCCCAAGCAA
SOX1	6656	GAACGCCTTCATGGTGTGGTC	TGTAATCCGGGTGCTCCTTCA
OTX2	5015	CCAGACATCTTCATGCGAGAG	GGCAGGTCTCACTTTGTTTTG
CORIN	10699	AATCCCACAACAGAGCATCG	GGAGCAGTTGACCTCGTCAG
FOXA2	3170	GGGGTAGTGCATCACCTGTT	CCGTTCTCCATCAACAACCT
MAP2	4133	CGAAGCGCCAATGGATTCC	TGAACTATCCTTGCAGACACCT
TH	7054	TGTCTGAGGAGCCTGAGATTCG	GCTTGTCCTTGGCGTCACTG
PITX3	5309	CCAACCTTAGTCCGTGCCAG	TGTGTAGGGCCTAGTCCACC
EN1	2019	TCTCGCTGTCTCTCCCTCTC	CGTGGCTTACTCCCCATTTA
EN2	2020	CCGGCGTGGGTCTACTGTA	CCTCTTTGTTCGGGTTCTTCTT
NURR1	4929	GCTGGACTCCCCATTGCTTT	CGGAGCTGTATTCTCCCGAA
LMX1A	4009	ACGTCCGAGAACCATCTTGAC	CACCACCGTTTGTCTGAGC
LMX1B	4010	CGGACTGCGCCAAGATGTT	TTGACTCGCATCAGGAAGCG
DDC	1644	ATTCATCTGCCCTGAGTTCCG	CCAATAGCCATTTGTGGGGAT
ALDH1A1	216	CCGTGGCGTACTATGGATGC	GCAGCAGACGATCTCTTTCGAT
ALDH1A2	8854	GGGTGTGTTCTTCAATCAAGGT	TGGTGGGGTCAAAGGGACT
GIRK2	3763	CACATCAGCCGAGATCGGAC	GGTAGCGATAGGTCTCCCTCA
KCNS3	3790	AGGAGCTGTGCGTATTCTCAT	CTTGCGTTCCTGGTAGCGATT
SOX6	55553	AGGGAGTCTTGCCGATGTG	CAGGCTCTCAGGTGTACCTTTA
OXR1	55074	TTCGACCAAACCTAAGTGATCCC	GGGGTGTCTAAACCTGTCATTG
SNCG	6623	TGAGCAGCGTCAACACTGTG	GAGGTGACCGCGATGTTCTC
SATB1	6304	AGAGCTGTCAGTGGAAGGAAACA	GTGGCACTGTTGAACGAAACAAAT
ZDHHC2	51201	TCCCGGTGGTGTTCATCAC	CAACTTGTTCGCCAGTGTTTTC
RAB3C	115827	GGAAGACGAGCGGGTCATC	CTCTCTGACATTTTGTCGCAGAT
FGF1	2246	ACACCGACGGGCTTTTATACG	CCCATTCTTCTTGAGGCCAAC
GAD1	2571	GCGGACCCCAATACCACTAAC	CACAAGGCGACTCTTCTCTTC
CALB1	793	TGTGGATCAGTATGGGCAAAGA	CTCAGTTTCTATGAAGCCACTGT
CALB2	794	AGCGCCGAGTTTATGGAGG	TGGTTTGGGTGTATTCCTGGA
TACR3	6870	CTCTGGTCCCTGGCGTATG	TGAAGCGCGTAGATGAAATTGA
CARTPT	9607	CCGAGCCCTGGACATCTACT	ATGGGAACACGTTTACTCTTGAG
VGF	7425	CGCTGACCCGAGTGAATCTG	CATACGCGCCTGGAATTGA
VIP	7432	CCAAAACAAACCAGAACAGTCAGC	TGAGAAGAGTCAGGAGCACAAGG
LPL	4023	TCATTCCCGGAGTAGCAGAGT	GGCCACAAGTTTTGGCACC
EFNB3	1949	CTCGGCGAATAAGAGGTTCCA	GTGAAGCGGAGATCCAGGTC
EGR1	1958	GGTCAGTGGCCTAGTGAGC	GTGCCGCTGAGTAAATGGGA
FZD1	8321	ATCGAAGCCAACTCACAGTATTT	CACGTTGTTAAGCCCCACG
ADCYAP1	116	CCACTCGGACGGGATCTTC	GCCGCCAAGTATTTCTTGACAG
SDC2	6383	TGGAAACCACGACGCTGAATA	ATAACTCCACCAGCAATGACAG
NEFM	4741	GAAATCGCTGCGTACAGAAAAC	TAATGGCTGTCAGGGCCTCTT
LRRK2	120892	TTTTGATGCCATGCACTCATTTC	GGAATCGCTAGGGAATGTAAACA
PARK2	5071	CCCACCTCTGACAAGGAAACA	TCGTGAACAAACTGCCGATCA
PARK7	11315	AACCGGAAGGGCCTGATAG	GCAAGAGGGTGTGTTGTAACT
SNCA	6622	AAGAGGGTGTTCTCTATGTAGGC	GCTCCTCCAACATTTGTCACTT
NCAM	4137	GGCTCCTTGGACTCATCTTTC	GACATCACCTGCTACTTCCTG
MAPT	4137	GAAGATTGGGTCCCTGGACAATA	AGGTCAGCTTGTGGGTTTCA
GAD2	2572	CAAACATTTATCAACATGCGCTTC	CTATGACACTGGAGACAAGGC
VGAT	140679	AGATGATGAGAAACAACCCCAG	CACGACAAGCCCAAAATCAC
DLX5	1749	ACAGAGACTTCACGACTCCCAG	TGTGGGGCTGCTCTGGTCTA
DLX6	1750	TGGTGAAAGAGAAGCATTTTGGACT	AGAGAAGGGCTGTTATGTGAGGAA
SERT	6532	TGCTGGCTTTTGCTAGCTAC	GAAGCTCGTCATGCAGTTCA
TPH2	121278	ATGGCTCAGATCCCCTCTACA	GGATCCGCAAGTAGTGGAACA
VGLUT1	57030	TCAAGTCCCCGATTCCGTGC	TGCGATTTTGGTTGTTTCCCCA
VGLUT2	57084	TGGGGCTACATCATCACTCA	GAAGTATGGCAGCTCCGAAA

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