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Biphasic Activation of WNT Signaling Facilitates the Derivation of Midbrain Dopamine Neurons from hESCs for Translational Use

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ABSTRACT

Human pluripotent stem cells show considerable promise for applications in regenerative medicine, including the development of cell replacement paradigms for the treatment of Parkinson's disease. Protocols have been developed to generate authentic midbrain dopamine (mDA) neurons capable of reversing dopamine-related deficits in animal models of Parkinson's disease. However, the generation of mDA neurons at clinical scale suitable for human application remains an important challenge. Here, we present an mDA neuron derivation protocol based on a two-step WNT signaling activation strategy that improves expression of midbrain markers, such as Engrailed-1 (EN1), while minimizing expression of contaminating posterior (hindbrain) and anterior (diencephalic) lineage markers. The resulting neurons exhibit molecular, biochemical, and electrophysiological properties of mDA neurons. Cryopreserved mDA neuron precursors can be successfully transplanted into 6-hydroxydopamine (6OHDA) lesioned rats to induce recovery of amphetamine-induced rotation behavior. The protocol presented here is the basis for clinical-grade mDA neuron production and preclinical safety and efficacy studies.

ATTACHMENTS

dinebiqa7.pdf

DOI

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PROTOCOL CITATION

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KEYWORDS

WNT Signaling, Midbrain Dopamine Neurons, hESCs, Biphasic Activation

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GUIDELINES

Materials Availability:

Cell lines generated and used in this study are available upon reasonable request from the Lead Contact.

Data and Code Availability:

The accession number for the RNA and ChIP sequencing data reported in this paper is GEO: GSE162884.

EXPERIMENTAL MODEL AND SUBJECT DETAILS:

Cell lines:

Human pluripotent stem cells [hPSCs; WA09 (H9; 46XX) and MEL1 (46XY)], EN1 knockout H9 hPSCs, and J1 human induced PSC (MRC5), which was previously published in Miller et al., (2013), were grown onto Vitronectin (VTN-N, Thermo Fisher #A14700) coated dishes with Essential 8 media (Life Technologies #A1517001). hPSCs were passaged every 4-5 days by EDTA, and passage 35-55 hPSCs were used for the experiments. For EN1 knockout in hPSCs, guide RNA was predicted with a top score from the CRISPR design tool (https://zlab.bio/guide-design-resources). Sequence of sgRNA for EN1 knockout was 5-AGCGATGGAGACAG CGTGC-3, and cloned into a CAG-Cas9 2A-GFP U6-sgRNA vector (Addgene, PX458) according to the published instruction (Ran et al., 2013). 5ug of plasmid was transfected to H9 hPSCs using Nucleofector (Lonza Kit V using the B-016 program). After 48h later, GFP expressed cells were FACS sorted using a BD FACS Aria III in the MSKCC Flow Cytometry core facility followed by growing clonally. Each colony was picked manually, genomic DNA was extracted, and validated EN1 knockout by DNA Sanger sequencing from amplified PCR product of the target region. PCR primers for this are 5-GCCGAGCATGGAAGAACA-3 and 5-CGGGTTCCCAGCTTTAGAC-3. All cell lines are cultured at **37 °C** with 5% CO2 and routinely tested for mycoplasma.

In vivo Animal studies:

Transplantation of hPSC-derived mDA neurons into nu/nu rat and NSG mice:

All procedures were performed following NIH guidelines and were approved by the local Institutional Animal Care and Use Committee (IACUC), the Institutional Biosafety Committee (IBC) and the Embryonic Stem Cell Research Committee (ESCRO). Female NIH nude (NIH-Foxn1rnu) rats were purchased from Taconic Biosciences. The animals were acclimated for at least five days to laboratory conditions before the procedures. 6-OHDA lesioning at 6-8 weeks old rats and cell transplantation were performed as described in Kriks et al. (2011). For cell transplantation, cells (450 000 cells/rat, 150 000/ml) were stereotaxic injected into right striatum at two deposit sites (1.5 ul/site) (AP: +1.0, ML: -2.5mm; VL: -4.7 and -4.4 mm; toothbar set at -2.5) of rat. Sham group received vehicle solution instead. For mouse studies, 6-8 weeks old NSG (NOD-SCID IL2Rgc$^{-/-}$) mice (Jackson Laboratory) were used, and a total of 2 mL cells (200,000/mouse) were injected at the speed of 0.5 ml/min into the dorsal striatum (AP +0.5, ML -1.8, DV -3.4 from dura) with the aid of stereotactic apparatus and electrical pump (Boston Scientific) to drive the syringe.

Amphetamine-induced rotation test:

Amphetamine-induced rotation test were performed before transplantation and once in a month after transplantation until 5 months post grafting. The rats were injected intraperitoneally of D-Amphetamine (Sigma, 5mg/kg). After 10 minutes, the rotation behavior was recorded 40 minutes and the total rotates were automatically counted by Ethovision XT 11.5 (Noldus Information Technology Inc., USA). The data were presented as (Ipsilateral-contralateral) rotates per minute.

MATERIALS TEXT

Antibodies:

Purified anti-Pax-6 **BioLegend Catalog #901301**

Anti-Tyrosine Hydroxylase Antibody clone LNC1 **Millipore Catalog #MAB318**

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Anti-LMX-1 Antibody **Millipore Catalog #AB10533**

OTX2 **Neuromics Catalog #GT15095**

Anti-MAP2 antibody (ab5392) **Abcam Catalog #ab5392**

Ab Rabbit TYROSINE HYDROXYLASE **Pel-Freez Catalog #P40101**

Monoclonal Mouse Anti-Human Ki-67 Antigen Clone MIB-1 **Agilent Technologies Catalog #M724001-2**

Human HNF-3 beta /FoxA2 Antibody **R&D Systems Catalog #AF2400**

Mouse monoclonal anti-EN1 **Developmental Studies Hybridoma Bank Catalog #4G11**

Calbindin D-28k **Swant Catalog #CB38**

ALDH1A1 Antibody (N-19) **Santa Cruz Biotechnology Catalog #sc-22588**

Anti-Synapsin I antibody produced in rabbit **Sigma Aldrich Catalog #S193**

Anti-Serotonin antibody produced in rabbit **Sigma Aldrich Catalog #S5545**

Anti-GIRK2 (Kir3.2) Antibody **Alomone Labs Catalog #APC-006**

Anti-Nuclei Antibody clone 235-1 **Merck Millipore Catalog #MAB1281**

EN1 Polyclonal Antibody **Thermo Fisher Scientific Catalog #PA5-14149**

Anti-trimethyl-Histone H3 (Lys27) Antibody **Merck Millipore Catalog #07-449**

Anti-Histone H3 (tri methyl K4) antibody - ChIP Grade **Abcam Catalog #ab8580**

Anti-Rabbit IgG HRP-Linked Whole Ab Donkey **Ge Healthcare Catalog #NA934**

Donkey anti-Goat IgG (H L) Secondary Antibody HRP **Invitrogen - Thermo Fisher Catalog #A15999**

Anti-Mouse IgG HRP-Linked Whole Ab Sheep **Ge Healthcare Catalog #NA931**

Donkey anti-Goat IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 **Thermo Fisher Scientific Catalog #A11055**

Donkey anti-Goat IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 **Thermo Fisher Scientific Catalog #A11057**

Donkey anti-Goat IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 **Thermo Fisher Scientific Catalog #A21447**

Citation: Tae Wan Kim, Jinghua Piao, So Yeon Koo, Sonja Kriks, Sun Young Chung, Doron Betel, Nicholas D. Socci, Se Joon Choi, Susan Zabierowski, Brittany N. Dubose, Ellen J. Hill, Eugene V. Mosharov, Stefan Irion, Mark J. Tomishima, Viviane Tabar, Lorenz Studer (08/19/2021). Biphasic Activation of WNT Signaling Facilitates the Derivation of Midbrain Dopamine Neurons from hESCs for Translational Use. https://dx.doi.org/10.17504/protocols.io.bu7znzp6

Donkey anti-Rabbit IgG (H L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 488 **Thermo Fisher Scientific Catalog #A-21206**

Donkey anti-Rabbit IgG (H L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 555 **Thermofisher Catalog #A-31572**

Donkey anti-Rabbit IgG (H L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 **Thermo Fisher Scientific Catalog #A31573**

Donkey anti-Mouse IgG (H L) ReadyProbes Secondary Antibody Alexa Fluor 488 **Thermo Fisher Scientific Catalog #R37114**

Donkey anti-Mouse IgG (H L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 555 **Thermofisher Catalog #A-31570**

Goat anti-Mouse IgG (H L) Alexa Fluor 647 **Thermo Fisher Scientific Catalog #A-21235**

Chemicals, Peptides, and Recombinant Proteins:

Vitronectin (VTN-N) Recombinant Human Protein, Truncated **Thermo Fisher Catalog #A14700**

TRIzol Reagent **Thermo Fisher Scientific Catalog #15596026**

Recombinant Human/Mouse FGF-8b Protein **R&D Systems Catalog #423-F8**

Accutase®, 100 ml **Innovative Cell Technologies, Inc Catalog #AT104**

UltrapPure 0.5M EDTA pH 8.0 **Invitrogen - Thermo Fisher Catalog #15575020**

L-Glutamine **Invitrogen - Thermo Fisher Catalog #25030-081**

Penicillin/Streptomycin **Invitrogen - Thermo Fisher Catalog #15140-122**

Essential 8™ Medium **Thermo Fisher Catalog #A1517001**

Neurobasal™ Medium **Thermo Fisher Catalog #21103049**

N2 Supplement-B 5 mL **Stemcell Technologies Catalog #7156**

B-27™ Supplement (50X), minus vitamin A **Thermo Fisher Catalog #12587010**

Y-27632 dihydrochloride (Rock Inhibitor) **R&D Systems Catalog #1254/10**

SB 431542 **R&D Systems Catalog #1614**

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Stemolecule LDN-193189 **Stemgent - Bio-connect Catalog #04-0074**

CHIR99021 **R&D Systems Catalog #4423**

Recombinant Mouse Sonic Hedgehog/Shh (C25II) N-Terminus **R&D Systems Catalog #464-SH**

Recombinant Human BDNF Protein **R&D Systems Catalog #248-BD**

L-ascorbic acid **Sigma**

Dibutyryl cAMP **Sigma Aldrich Catalog #D0627**

GDNF (Glial cell line-Derived Neurotrophic Factor) **peprotech Catalog #450-10**

TGFβ3 (recombinant human transforming growth factor-beta 3) **R&D Systems Catalog #243-B3**

DAPT **R&D Systems Catalog #2634**

Poly-L-Ornithine (PLO) **Sigma Aldrich Catalog #P3655**

Fibronectin **Corning Catalog #356008**

Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix **Thermo Fisher Catalog #A1413201**

STEM-CELLBANKER - GMP Grade **amsbio Catalog #11890**

DAPI **Sigma Aldrich Catalog #D9542**

Critical Commercial Assays:

SsoFast™ EvaGreen® Supermix 1000 x 20 µl rxns 10 ml (10 x 1 ml) **Bio-rad Laboratories Catalog #1725202**

iScriptTM cDNA synthesis kit **BIO-RAD Catalog #170-8841**

SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) **Cell Signaling Technology Catalog #9005**

Perm/Wash Buffer **BD Biosciences Catalog #554723**

Pierce™ BCA Protein Assay Reagent A **Thermo Fisher Catalog #23228**

Recombinant DNA:

pSpCas9(BB)-2A-GFP (PX458) **addgene Catalog #48138**

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A	B	C
REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
Antibodies		
Rabbit polyclonal anti-PAX6	Biolegend	Cat#901301; RRID: AB_256503
Mouse monoclonal anti-Tyrosine Hydroxylase, clone LNC1	Millipore	Cat#MAB318; RRID:AB_2201528
Rabbit polyclonal anti-LMX-1	Millipore	Cat#AB10533; RRID: AB_10805970
Goat Anti-OTX2	Neuromics	Cat#GT15095-100; RRID: AB_2157174
Chicken polyclonal anti-MAP2	Abcam	Cat# ab5392; RRID:AB_2138153
Rabbit polyclonal anti-TH	Pel-Freez Biologicals	Cat#P40101-150; RRID:AB_2617184
Mouse monoclonal anti-Ki-67 antigen (clone MIB-1)	Agilent	Cat# M724001-2, RRID:AB_2631211
Goat polyclonal anti-FOXA2	R&D Systems	Cat# AF2400; RRID:AB_2294104
Mouse monoclonal anti-NURR1	Perseus Proteomics	Cat# PP-N1404-00; RRID:AB_2251476
Mouse monoclonal anti-EN1	DSHB	Cat#4G11; RRID: AB_528219
Rabbit polyclonal anti-Calbindin D-28k	Swant	Cat#CB38; RRID: AB_2721225
Mouse monoclonal anti-STEM121	Takara Bio Inc	Cat#AB-121-U-050; RRID: AB_2632385
Goat anti-ALDH1A1	Santa Cruz Biotechnology	Cat#sc-22588; RRID:AB_2289311
Rabbit polyclonal anti-Synapsin I	Sigma-Aldrich	Cat#S193; RRID:AB_261457
Rabbit polyclonal anti-Serotonin (5-HT)	Sigma-Aldrich	Cat#S5545; RRID: AB_477522
Rabbit polyclonal anti-GIRK2	Alomone labs	Cat#APC-006; RRID:AB_2040115
Mouse monoclonal anti-human nuclei	Millipore	Cat#MAB1281; RRID: AB_94090
Rabbit polycolnal anti-EN1	Thermo Fisher Scientific	Cat#PA5-84917; RRID:AB_2792066
Rabbit polyclonal anti-H3K27me3	Millipore	Cat#07-449; RRID:AB_310624
Rabbit polyclonal to Histone H3 (tri methyl K4)	Abcam	Cat#ab8580; RRID:AB_306649
HRP-linked donkey anti–rabbit IgG	GE Healthcare	Cat# NA934; RRID:AB_772206
HRP-linked anti-Goat IgG (H+L)	Invitrogen	Cat# A15999; RRID:AB_2534673
HRP-linked sheep anti–mouse IgG	GE Healthcare	Cat: NA931; RRID:AB_772210
AlexaFluor Donkey Anti-Goat 488	Thermo Fisher Scientific	Cat#A-11055; RRID: AB_2534102
AlexaFluor Donkey Anti-Goat 568	Thermo Fisher Scientific	Cat#A-11057; RRID: AB_142581
AlexaFluor Donkey Anti-Goat 647	Thermo Fisher Scientific	Cat#A-21447; RRID: AB_141844
AlexaFluor Donkey Anti-Rabbit 488	Thermo Fisher Scientific	Cat#A-21206; RRID: AB_141708
AlexaFluor Donkey Anti-Rabbit 555	Thermo Fisher Scientific	Cat#A-31572; RRID: AB_162543
AlexaFluor Donkey Anti-Rabbit 647	Thermo Fisher Scientific	Cat#A-31573; RRID: AB_2536183
AlexaFluor Donkey Anti-Mouse 488	Thermo Fisher Scientific	Cat#R37114; RRID: AB_2556542
AlexaFluor Donkey Anti-Mouse 555	Thermo Fisher Scientific	Cat#A-31570; RRID: AB_2536180
AlexaFluor Donkey Anti-Mouse 647	Thermo Fisher Scientific	Cat#A-21235; RRID: AB_141693
Chemicals, Peptides, and Recombinant Proteins		
Vitronectin (VTN-N)	Thermo Fisher Scientific	A14700

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Trizol	Thermo Fisher Scientific	15596026
Recombinant human FGF8b	R&D	423-F8
Accutase	Innovative Cell Technologies	AT104-500
0.5M EDTA, pH 8.0	Thermo Fisher Scientific	15575-020
L-Glutamine (100X)	Thermo Fisher Scientific	25030-081
Penicillin Streptomycin	Thermo Fisher Scientific	15140-122
Essential 8 (E8)	Thermo Fisher Scientific	A1517001
Neurobasal	Life Technologies	21103-049
N2 supplement B	Stem Cell Technologies	7156
B27	Life Technologies	12587-010
Y-27632 (ROCKi)	R&D	1254
SB431542 (SB)	R&D	1614
LDN193189 (LDN)	Stemgent	04-0074
CHIR99021	R&D	4432
SHH C25II	R&D	464-SH
brain-derived neurotrophic factor (BNDF)	R&D	248-BD
ascorbic acid (AA)	Sigma	4034
dibutyryl cAMP (cAMP)	Sigma	4043
glial cell line-derived neurotrophic factor (GDNF)	Peptrotech	450-10
transforming growth factor type b3 (TGFb3)	R&D	243-B3
DAPT	R&D	2634
Poly-L-Ornithine (PO)	Sigma Aldrich	P3655
Mouse Laminin I (LAM)	R&D	3400-010-1
Fibronectin (FN)	Thermo Fisher Scientific	356008
Geltrex	Life Technologies	A1413201
STEM-CELLBANKER	Amsbio	11890
4% paraformaldehyde	Affymetrix	MFCD00133991
40, 6-diamidino-2-phenylindole (DAPI)	Sigma	D9542
Critical Commercial Assays		
RNA MiniPrep kit	Zymo Research	R2052
SsoFast EvaGreen Supermix	Bio-Rad	172-5202
iScript Reverse Transcription Supermix	Bio-Rad	170-8841
SimpleChIP Plus Enzymatic Chromatin IP Kit	Cell signaling Tech	9005
BD Perm/Wash Buffer	BD Biosciences	554723
BCA protein assay kit	Pierce	23228
Deposited Data		
RNA-Seq	This study	GEO: GSE162884
ChIP-Seq	This study	GEO: GSE162884
Experimental Models: Cell Lines		
Human: H9 (WA-09) hESC line	WiCell Research Institute	NIHhESC-10-0062

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MEL-1 hESC line	Stem Cells Ltd	NIHhESC-11-0139
MRC5 (J1) iPSC line	MSKCC Stem Cell Core	Miller et al., 2013
Oligonucleotides		
See Table S1		N/A
Recombinant DNA		
PX458 Cas9-GFP	Addgene	Addgene: 48138
Software and Algorithms		
DESeq2	Love et al., 2014	http://bioconductor.org/packages/release/bioc/html/DESeq2.html
R	https://cran.r-project.org/	N/A
Bowtie2	(Langmead and Salzberg, 2012)	http://bowtie-bio.sourceforge.net/bowtie2
STAR aligner (v.2.4.2a)	(Dobin et al., 2013)	https://github.com/alexdobin/STAR
FLEXBAR (v.2.2)	(Dodt et al., 2012)	https://github.com/seqan/flexbar
FeatureCounts (v.1.4.2)	(Liao et al., 2014)	http://bioinf.wehi.edu.au/featureCounts/
MACS	(Zhang et al., 2008)	https://github.com/taoliu/MACS
CRISPR design tool	(https://zlab.bio/guide-designresources)	N/A
FIJI - ImageJ	(Schindelin et al., 2012)	https://fiji.sc/
FlowJo 9	https://www.flowjo.com	N/A
Integrative Genomics Viewer (IGV)	Robinson et al., 2011	http://software.broadinstitute.org/software/igv/
Picardtools (version 2.9.5) Broad Institute http://broadinstitute.github.io/picard/	Broad Institute	http://broadinstitute.github.io/picard/

Directed differentiation into midbrain dopamine neurons (mDA) 50m

1 Dissociate hPSCs into single cells using Accutase (Cell Technologies, #AT104), and plate at 400K cells/cm^2 onto Geltrex (Life Technologies, #A1413201) coated dishes with Neurobasal (Life Technologies)/N2 (Stem Cell Technologies)/B27(Life Technologies) media containing [M]**2 Milimolar (mM)** L-glutamine, [M]**500 ng/ml** SHH C25II (R&D systems #464-SH), [M]**250 Nanomolar (nM)** LDN (Stemgent # 04-0074-02), [M]**10 Micromolar (µM)** SB431542 (R&D systems #1614), [M]**0.7 Micromolar (µM)** CHIR99021 (R&D systems #4432), and [M]**10 uM** Rock inhibitor (Y-27632, R&D systems #1254), which represents day 0 of differentiation, and culture until day 3 without Rock inhibitor from day 1.

2 On day 4, expose cells to different concentration of CHIR [M]**0.7 Micromolar (µM)** , [M]**3 Micromolar (µM)** , [M]**5 Micromolar (µM)** , and [M]**7.5 Micromolar (µM)** until day 10.

3 On day 7, withdraw LDN, SB, and SHH.

4 On day 10, change media to Neurobasal/B27/L-Glu supplemented with BDNF (brain-derived neurotrophic factor, [M]**20 ng/ml** ; R&D #248-BD), ascorbic acid ([M]**0.2 Milimolar (mM)** , Sigma #4034), GDNF (glial cell line-derived neurotrophic factor, [M]**20 ng/ml** ; Peprotech # 450-10), TGFb3 (transforming growth factor type b3, [M]**1 ng/ml** ; R&D #243-B3), dibutyryl cAMP ([M]**0.2 Milimolar (mM)** ; Sigma #4043), and CHIR [M]**3 Micromolar (µM)** .

5 On day 11, dissociate cells using Accutase and replate under high cell density (800K cells/cm^2) on polyornithine (PO;

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[M]**15 µg/ml**)/ laminin ([M]**1 µg/ml**)/ fibronectin ([M]**2 µg/ml**) coated dishes in mDA differentiation media [(NB/B27/L-Glu, BDNF, ascorbic acid, GDNF, dbcAMP, and TGFb3 until day 16 with adding DAPT ([M]**10 Micromolar (µM)** R&D #2634)] from day 12.

6 On day 16, dissociate cells and plate as same procedure of day 11 and culture until day 25 using mDA differentiation media.

7 On day 25, dissociate cells using Accutase and replate under low cell density (200K~300K cells/cm^2) in mDA differentiation media until the desired experiments.

8 For the cryopreservation of mDA precursor neurons, treat day16 mDA differentiated cells with Accutase for **00:20:00** - **00:30:00** , washing, detached, single cells, and pelleting. 50m

9 Resuspend cell pellets at a cell density of 8 million cells/mL of STEMCELLBANKER.

10 Use controlled rate freezer (ThermoFisher) to cryopreserve cell product.

Immunohistochemistry 1h 30m

11 Fix cells in 4% paraformaldehyde (PFA) (Affymetrix #MFCD00133991) in DPBS for **00:15:00** at **Room temperature** . 15m

12

Wash cells subsequently with DPBS.

13 Permeabilize the samples with 0.5% Triton X-100 and block with 2% BSA in DPBS.

14 15m

Incubate the samples with primary antibody **Overnight** at **4 °C** .

15 1h

The next day, after washing with DPBS, incubate the samples with secondary antibody conjugated with Alexa Fluor 488- 555-, or 647- (Thermo Fisher) diluted at 1:400 in 2% BSA (DPBS) for **01:00:00** at **Room temperature** .

16

Wash the samples with DPBS and count-stain with 40, 6-diamidino-2-phenylindole (DAPI) (Sigma, #D9542).

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17

Visualize the images using an Olympus and Zeiss inverted fluorescence microscope.

18 Use Mouse and chicken anti-MAP2 (1:1500, Sigma and 1:2000, Abcam), rabbit and mouse anti-TH (1:500, PelFreez and 1:1000, Immunostar), goat anti-FOXA2 (1:200, R&D), Rabbit anti-LMX1A (1:1500, Abcam), Goat anti-OTX2 (1:1000, Neuromics), rabbit and mouse anti-PAX6 (1:500, Covance and 1:200, BD-Biosciences), mouse and rabbit anti-EN1 (1:50, DSHB and 1:200 Invitrogen), goat anti-ALDH1A1 (1:250, Santa Cruz), rabbit anti-GIRK2 (1:400, Almonte), rabbit anti-CALB1 (1:2000 Swant), and mouse anti-NURR1 (1:1500, Perseus Proteomics) for immuno-fluorescent staining.

19 Use donkey anti- mouse, goat, rabbit or chicken secondary antibodies conjugated with Alexa Fluor-488, Alexa Fluor-555 or Alexa Fluor-647 fluorophore (1:400, Life technologies). Counterstain nuclei by DAPI.

Western blotting 3h

20 Collect and lyse the cultured cells and with 2X Laemmli Sample Buffer (Bio-Rad, #161-737).

21 After protein quantification using BCA protein assay kit (Pierce, #23228), load and separate the same amount of proteins from samples by NuPAGE 4%–12% Bis-Tris Protein Gel (Invitrogen, #NP0322BOX) using NuPAGE MES SDS Running Buffer (Invitrogen, #NP0060).

22 Transfer proteins electrophoretically to a nitrocellulose membrane using NuPAGE Transfer Buffer (Invitrogen, #NP0006) with 20% Methanol.

23 2h

Block the membranes in 5% skim milk (TBS-T) for **01:00:00** at **Room temperature** and incubate primary antibodies **Overnight** at **4 °C** .

24 1h

After washing with TBS-T, incubate secondary mouse or rabbit antibodies conjugated to horseradish peroxidase for **01:00:00** at **Room temperature** .

25 After three times washing, perform developing the signals by using an enhanced chemiluminescence (ECL) detection kit (PerkinElmer, #NEL104001WA).

RNA extraction and Real-time qRT-PCR

26 Isolate the total RNAs from samples with TRIzol (QIAGEN) using the Direct-zol RNA MiniPrep kit (Zymo Research, #R2052).

27 Use **1 µg** of RNA to generate cDNA using the iScript Reverse Transcription Supermix (BioRad, #170-8841).

28

Perform real-time qRT-PCR using the SSoFAST EvaGreen Mix (BioRad) in a BioRad CFX96 Thermal Cycler.

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29 Perform all reactions according to the manufactured protocol.

30 Obtain some primers from QIAGEN (Quantitect Primer assays). Normalize results to GAPDH.

Primer sequences are listed in Table S1.

RNA-sequencing

31 Perform RNA-seq library preparation at the MSKCC Integrated Genomics Operation Core Facility.

32 Sequence libraries on an Illumina HiSeq 2500 platform with 50bp paired end reads.

33 Filter the sequencing data for quality and remove adaptor sequences using Flexbar (v.2.2) (Dodt et al., 2012) and align to hg19 using STAR aligner (v.2.4.2a) (Dobin et al., 2013).

On average, we obtain ~50M reads per sample with > 97% mapped reads.

34 Generate gene read coverage using FeatureCounts (v.1.4.2) (Liao et al., 2014) using GENCODE annotation (v19) (Harrow et al., 2012).

35 Perform differential gene expression using DESeq2 (v. 1.12.4) (Love et al., 2014) and annotate using biomaRt package (v. 2.28) (Durinck et al., 2009).

ChIP-sequencing

36 Perform chromatin immune-precipitation (ChIP) for H3K27me3 (Millipore, #07-449) and H3K4me3 (Abcam, #ab8580) from each sample using SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell signaling Tech, #9005) according to the instructions of the manufacturer.

37 Generate ChIP-sequencing library at the MSKCC Integrated Genomics Operation Core Facility.

38 Sequence the libraries on an Illumina HiSeq 2500 platform with 50bp paired end reads.

39 Process the generated each FASTQ files to remove any adaptor sequences at the end of the reads using cutadapt (v1.6).

40 Map the files using the BWA mapper (bwa mem v0.7.12).

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41 Sort the SAM files after mapping and add read group tags using the PICARD tools.

42 After sorting in coordinate order, process the BAM's with PICARD MarkDuplicates.

43 Do peak calling using the MACS program (Version 2).

Electrophysiological recordings

44 Perform patch-clamp electrophysiological recording on hPSC-derived mDA neurons plated on a monolayer of rat cortical astrocytes, as described previously (Rayport et al., 1992).

45 Conduct recording at day 40, 60, and 75 on randomly selected neurons at **Room temperature** in a Tyrode's solution containing (in mM): **119 Milimolar (mM)** NaCl, **3 Milimolar (mM)** KCl, **10 Milimolar (mM)** glucose, **2 Milimolar (mM)** $CaCl_2$, **1.2 Milimolar (mM)** $MgCl_2$-6 H_2O, **3.3 Milimolar (mM)** HEPES, and **2.7 Milimolar (mM)** HEPES-Na^+ salt (**pH7.4** , 270 mOsm).

46 For whole-cell patch-clamp studies, pull borosilicate glass pipettes (G150F-4, Warner Instruments) with a tip resistance of 3-4 MΩ on a P-97 Flaming-Brown micropipette puller (Sutter Instruments) and fill with (in mM): **115 Milimolar (mM)** K-gluconate, **20 Milimolar (mM)** KCl, **10 Milimolar (mM)** HEPES, **2 Milimolar (mM)** $MgCl_2$, **2 Milimolar (mM)** ATP-Mg, **2 Milimolar (mM)** ATP-Na_2 and **0.3 Milimolar (mM)** GTP-Na, (**pH7.25** , ~280 mOsm).

47 

Visualize neurons under a 40x water immersion objective using Olympus BX51W1 microscope (Olympus), and perform recording with an Axopatch 700B amplifier (Molecular Devices) and digitize at 10 kHz with ITC-18 (HEKA Instruments Inc).

48 Acquire data using WinWCP software (John Dempster, University of Strathclyde, UK).

49 In each cell, monitor input resistance (measured by -100 pA, 1 s hyperpolarizing pulse), resting membrane potential and spontaneous firing frequency throughout the recording.

50 Measure current-voltage relationship and evoked action potentials by injecting a 1 s long somatic current from -30 to +20 pA in +10 pA increments and from 0 to +250 pA in +10 pA increments, respectively.

51 To measure HCN currents, hold cells at -50 mV in voltage-clamp mode and apply hyperpolarizing voltage steps from -70 to -160 mV.

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52 Measure KCNQ currents at -30 mV holding potential with -30 to -70 mV hyperpolarizing voltage range.

53 Induce sodium and slow potassium currents by a depolarizing voltage step from 0 to +110 mV.

54 Perform data analysis and statistics using Clampfit (Molecular Devices) and GraphPad Prism (GraphPad software).

Data are presented as mean ± SEM.

HPLC 55m

55 For DA measurement experiments, plate mDA neurons onto PO/laminin/fibronectin coated 24-well plates in 5×10^5 cells on day 25 and use between day 60 and day 75.

HPLC with electrochemical detection (HPLC-EC) was done as previously described (Pothos et al., 1996).

56 30m

Briefly, prior to supernatant collection, incubate cells in fresh DMEM: F12 + N2 media for **00:30:00** .

57 10m

After exposure to either Tyrode's saline alone or supplemented with high KCl (**40 Milimolar (mM)** , Sigma) for **00:10:00** at **Room temperature** , collect the supernatant and mix immediately with perchloric acid (**0.1 Molarity (M)** final concentration) to deproteinize the sample and prevent dopamine auto-oxidation.

58 15m

Sonicate the samples at **Room temperature** for **00:10:00** , centrifuge at **10000 x g** for **00:05:00**), store at **-80 °C** and analyze within the following two weeks by reverse phase HPLC-EC.

59 Collect the cells in each sample to normalize for protein content.

60 Normalize DA concentrations in each group of samples to the levels in the corresponding control group.

Data are shown as averaged normalized values from 2 independent experiments.

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