Deriving dopaminergic neurons for clinical use. A practical approach.

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Supplementary Information

Methods

RT-PCR analysis

Total RNA from at least triplicate samples with around 1 million cells each was isolated using either QIAsymphony automatic purification system or RNeasy Plus Mini kit, according to manufacturer's instructions (Qiagen). Total RNA was used for reverse transcription with the iScript cDNA synthesis kit (Biorad) and Px2 Thermal Cycler (Thermo Scientific). To analyze gene expression, PCR reactions were performed in duplicate using 1/25-th of the cDNA per reaction and the QuantiTect Primer Assay and Quantitest SYBR Green master mix (Qiagen). qPCR was performed using the Rotor-Gene Q (Qiagen) 5 min at 95°C, 5 s at 92°C, and 20 s at 60°C for 37 cycles followed by melt to check the specificity of the amplicons from 50 to 99°C raising by 1°C each step. Relative quantification was performed against a standard curve and quantified values were normalized against the input determined by PPIG. The primers used for the analysis are listed in Supplementary Table S1. The results are presented as presented as mean ± s.e.m. and statistical analysis was performed using a confidence level of 95% ($\alpha = 0.05$) with the two-tailed Student's *t*-test for comparing two groups or one-factor ANOVA with Dunnett test for comparing multiple groups against control and $P < 0.05$ was considered significant.

Immunocytochemistry

Around 100,000 cells per sample were fixed with 4% paraformaldehyde for 10 min at room temperature, washed with PBS, and permeabilized and blocked for 1 hour at room temperature in 0.3% Triton X-100, 5% normal donkey serum, and 1% BSA in PBS. The cells were incubated overnight at 4°C with primary antibodies in 0.3% Triton X-100, 2% BSA in PBS. Cells are washed three times with PBS and incubated for 1 hour at room temperature with secondary antibodies in 0.3% Triton X-100, 5% normal donkey serum, and 1% BSA in PBS. The nuclei

were stained with DAPI. All antibodies used are listed in Supplementary Table S2. Representative images are shown from at least three independent experiments.

Flow cytometry

For flow cytometry analysis, 1 million cells per sample were harvested with Accutase, washed with PBS, and fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were washed twice with PBS and blocked for 1 hour at room temperature with 0.3% Triton X-100, 5% normal donkey serum, and 1% BSA in PBS. Cells were then incubated overnight at 4°C with primary antibody in 0.3% Triton X-100, 5% normal donkey serum, and 1% BSA in PBS. Cells were washed twice with PBS and incubated for 1 hour at room temperature with secondary antibody in .3% Triton X-100, 5% normal donkey serum, and 1% BSA in PBS. The samples were run on a Becton Dickinison FACSCalibur™ 4-color flow cytometer and the data was analyzed with CellQuest Pro™ software (v6.0). The antibodies used are listed in Supplementary Table S2. Representative results are shown from three independent experiments.

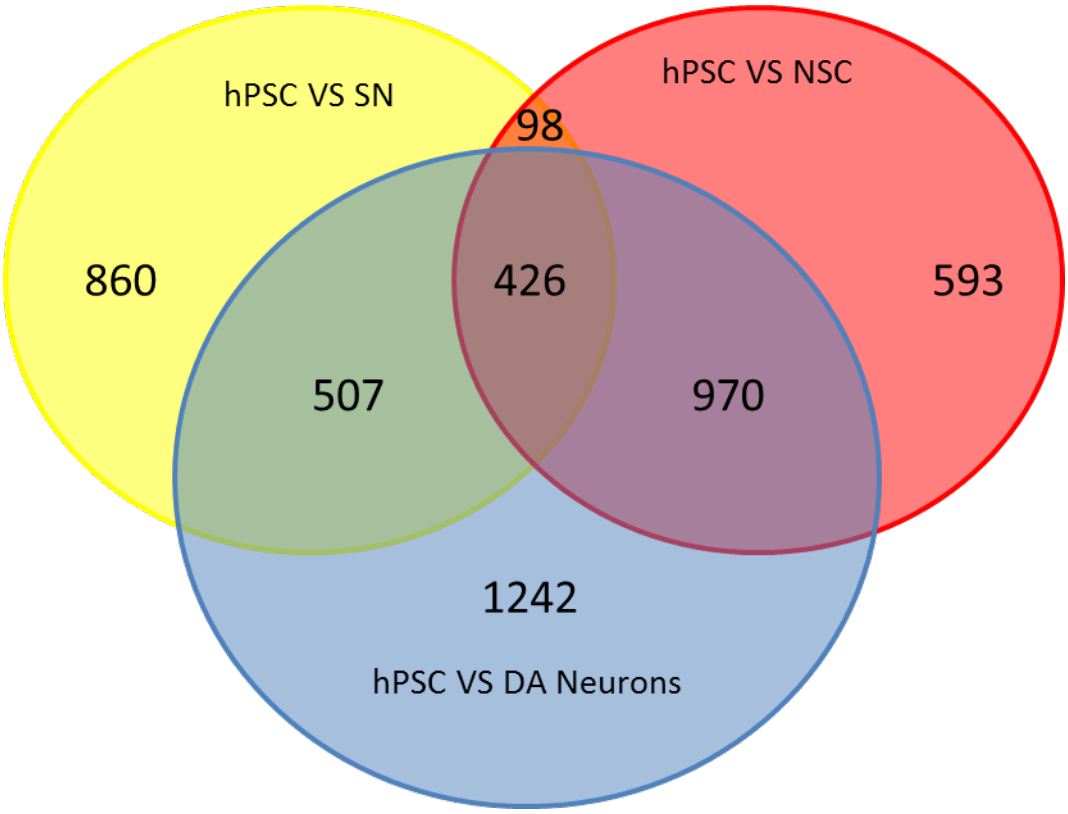
Microelectrode array (MEA) system

Extracellular voltage recordings from cultured hPSC-derived DA neurons were made on day 95 of differentiation in NB medium [NeuroBasal medium, 1X GlutaMAX, 1X N2/B27 Supplement (Invitrogen)] supplemented with 2.5 µM guggulsterone at 37°C using a Muse microelectrode array (MEA) system (Axion Biosystems). The surface of the MEA dishes contained an 8 x 8 grid (64 electrodes total) of 30 µm diameter round extracellular electrodes composed of nanoporous platinum, with 200 µm inter-polar spacing.

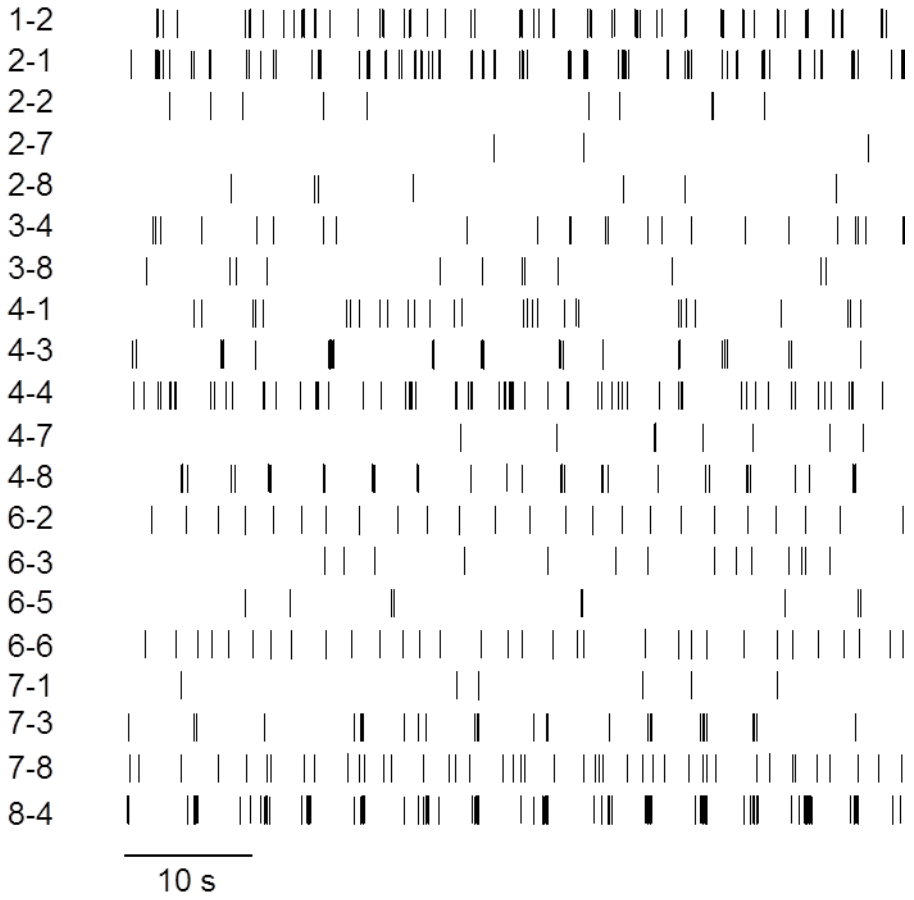
MEA dishes were sterilized by rinsing 3 times in sterile deionized water, once in 70% ethanol and once in 100% ethanol. Dishes were then capped and inverted in a covered petri dish, and

baked at 50˚C for 4-5 hr. A polyethyleneimine (PEI) base coating was deposited by adding 500 ml of filtered (0.22 μm filter) 0.1% PEI in sodium borate buffer (15 mM; pH 8.4) to each dish and incubating at room temperature for 1 hr. Immediately following aspiration of the PEI solution, dishes were washed 4 x with 1 ml deionized water, and air-dried overnight in a tissue culture hood. 75 μl of a sterile Matrigel solution diluted 1:30 in KnockOut DMEM/F12 was added directly over the electrode grid, and the dish was incubated for 1 hr at 37°C in a tissue culture incubator. The Matrigel solution was then removed and 50 μl of 2.5 μM guggulsterone in NB media containing approximately 100,000 cells were plated onto the surface of the MEA and incubated at 37°C for 30 min to allow cell attachment. The DA neurons had been previously cultured on Matrigel in 2.5 μM guggulsterone in NB media for 65 days and were removed from the plate by mechanical trituration. After the 30 min incubation, 550 μl of NB media was added to the MEA well and incubated at 37°C for 24 hours. Media was exchanged and then every 3-4 days thereafter.

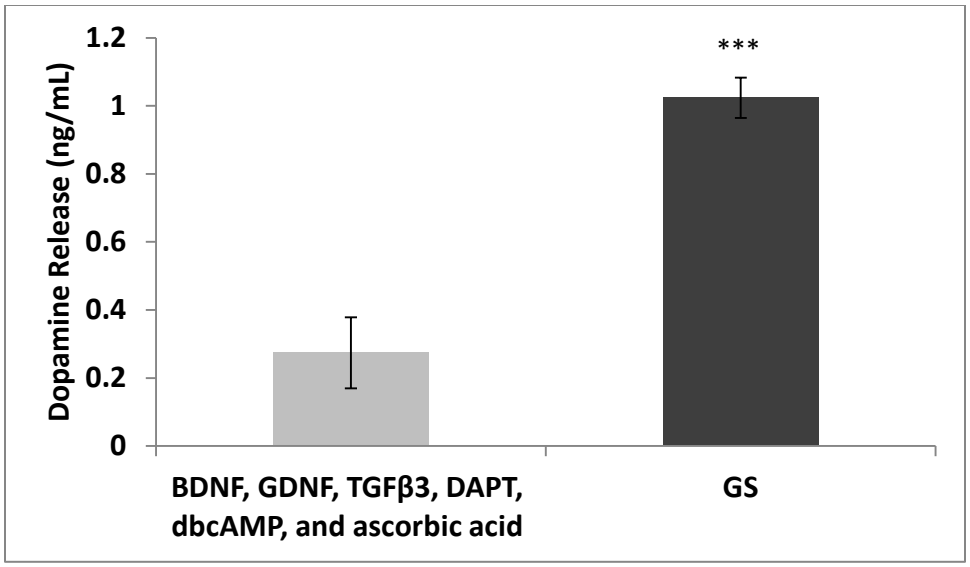
Voltage data were sampled simultaneously on all electrodes at 12.5 kHz with a hardware filter bandwidth of 200-5000 Hz, and saved to computer with AxIS software (Axion Biosystems). To remove high-frequency electrical noise before neuronal spike detection, raw signals were treated in software with a 200-2500 Hz single-order Butterworth band-pass filter. Action potential spikes were identified with a detection threshold set to ± 5 times standard deviation of the baseline electrode noise. This threshold generally equaled between 7.5 and 11.25 μV. Color-coded spike rate maps were made with AxIS. For spike raster plots and inter-spike interval histograms, time-stamps of detected spikes were exported to Neuroexplorer (NEX Technologies). Two independent recordings were performed.



Supplementary Figure S1. Venn diagram showing the similarities between three different groups of probes: hPSC vs Substantia Nigra (SN) (yellow), hPSC vs NSC (red), and hPSC vs DA Neurons (blue). The majority of the genes detected comparing hPSC vs SN and hPSC vs adult NSC are also common to the comparison between hPSC vs SN and hPSC vs DA Neurons. The number of probes common in the comparisons hPSC vs NSC and hPSC vs DA neurons (1396) and hPSC vs DA neurons and hPSC vs SN (933) is very similar, suggesting that DA neurons are in an intermediate state between adult NSCs and SN. The majority of the genes that overlap in hPSC vs NSC and hPSC vs SN comparison are likely to be responsible to carry a specific signature associated with NSCs. Most of these genes (426) are also present in DA neurons suggesting that DA neurons acquired the specific signature necessary in the neuronal differentiation process. This suggests again that DA neurons are developmentally located after NSCs and before the SN stage.



Supplementary Figure S2. MEA recordings reveal many active neurons in cultures of guggulsterone-treated neurons. 60 s spike raster plots for all electrodes in an MEA dish with spike rate ≥ 5 spikes/min, averaged over 10 min. Each detected spike is represented by one hash mark. Physical layout of electrodes is given in Supplementary Video S1.



Supplementary Figure S3. Comparison of DA released in vitro between DA neurons derived with GS versus BDNF, GDNF, TGFβ3, DAPT, dbcAMP, and ascorbic acid. DA neurons derived in the last step with GS secreted three times higher DA levels *in vitro*. Mean ± s.d., two-tailed Student's *t*-test: n = 3-10; $\alpha = 0.05$; ***$P < 0.001$.

Supplementary Video S1. Movie of spike activity created with AxIS software (Axion Biosystems). Spike rate was linearly interpolated between values calculated every 0.5 s. Intensity scale is given at right.

Supplementary Table S1: RT-PCR primers

Gene	Catalog #	Manufacturer
TH	QT00067221 QuantiTect Primer Assay	Qiagen
KCNJ6 (GIRK2)	QT00010444 QuantiTect Primer Assay	Qiagen
SLC18A2 (VMAT2)	QT00059857 QuantiTect Primer Assay	Qiagen
MAP2	QT00057358 QuantiTect Primer Assay	Qiagen
MSI1	QT00025389 QuantiTect Primer Assay	Qiagen
NES	QT00235781 QuantiTect Primer Assay	Qiagen
PAX6	QT00071169 QuantiTect Primer Assay	Qiagen
POU5F1	QT00210840 QuantiTect Primer Assay	Qiagen
DDC	QT00046774 QuantiTect Primer Assay	Qiagen
SOX1	QT01008714 QuantiTect Primer Assay	Qiagen
SOX2	QT00237601 QuantiTect Primer Assay	Qiagen
SLC6A3 (DAT)	QT00000231 QuantiTect Primer Assay	Qiagen
LMX1B	QT00025746 QuantiTect Primer Assay	Qiagen
PAX5	QT00021399 QuantiTect Primer Assay	Qiagen
NR4A2 (NURR1)	QT00037716 QuantiTect Primer Assay	Qiagen
NANOG	QT01844808 QuantiTect Primer Assay	Qiagen
EN1	QT00033299 QuantiTect Primer Assay	Qiagen
FOXA2	QT00212786 QuantiTect Primer Assay	Qiagen
TUBB3	QT00083713 QuantiTect Primer Assay	Qiagen
PPIG (Cyclophilin G)	QT01676927 QuantiTect Primer Assay	Qiagen

Supplementary Table S2: Antibodies

Antigen	Catalog #	Dilution	Application	Manufacturer
TUBB3	MAB1637	1:100	ICC	Millipore
MSI1	ab52865	1:300	ICC	Abcam
MSI1	ab52865	1:40	FACS	Abcam
PAX6	PRB-278P	1:100	ICC	Covance
PAX6	PRB-278P	1:20	FACS	Covance
NES	ab6320	1:200	ICC	Abcam
NES	ab6320	1:40	FACS	Abcam
TH	P40101	1:100	ICC	Pel-Freeze
TH	ab75875	1:40	FACS	Abcam
NURR1	sc-991	1:100	ICC	Santa Cruz Biotechnology, Inc.
GIRK2	ab30738	1:300	ICC	Abcam
HNF-3β (FOXA2)	sc-6654	1:200	ICC	Santa Cruz Biotechnology, Inc.
DAT	sc-14002	1:100	ICC	Santa Cruz Biotechnology
Rabbit polyclonal IgG-ChIP Gade	ab27478	1:40	FACS	Abcam