Supplementary Methods

Data presented throughout the manuscript are generated from differentiations performed on matrigel coated plates without the optional sorting step and using the WA09 cell line unless stated otherwise. All hPSC lines used in these studies have undergone regular mycoplasma testing and STR profiling to ensure quality and authenticity prior to differentiations.

Materials

REAGENTS - qRT-PCR

- RNeasy RNA purification kit (Qiagen, 74106)
- SYBR™ Green PCR Master Mix (Applied Biosystems, 4309155)
- Superscript IV Reverse Transcriptase Kit (Invitrogen, 18090010)
- RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, 10777019)
- Random Primers (Invitrogen, 48190011)
- dNTPs for cDNA Probe Synthesis (10 mM) (Invitrogen, AM8200)
- Hs_SOX10_1_SG QuantiTect Primer Assay (Qiagen, QT0005540)
- Hs_EDNRB_1_SG QuantiTect Primer Assay (Qiagen, QT00014343)
- Hs_PHOX2A_1_SG QuantiTect Primer Assay (Qiagen, QT00215467)
- Hs_PHOX2B_1_SG QuantiTect Primer Assay (Qiagen, QT00015078)
- Hs_HAND2_2_SG QuantiTect Primer Assay (Qiagen, QT01012907)
- Hs_ASCL1_1_SG QuantiTect Primer Assay (Qiagen, QT00237755)
- Hs_NTRK3_1_SG QuantiTect Primer Assay (Qiagen, QT00052906)
- Hs_ASLC6A4_1_SG QuantiTect Primer Assay (Qiagen, QT00058380)
- Hs_CHAT_1_SG QuantiTect Primer Assay (Qiagen, QT00029624)
- Hs_SERT_1_SG QuantiTect Primer Assay (Qiagen, QT0058380)
- Hs_NOS1_1_SG QuantiTect Primer Assay (Qiagen, QT00043372)
- Hs_TUBB_1_SG QuantiTect Primer Assay (Qiagen, QT00089775)
- Hs_GFAP_1_SG QuantiTect Primer Assay (Qiagen, QT00081151)
- Hs_GAPDH_1_SG QuantiTect Primer Assay (Qiagen, QT00079247)

REAGENTS – IMMUNOCYTOCHEMISTRY AND FLOW CYTOMETRY

- PFA, Paraformaldehyde Solution 4% in PBS (Alfa Aesar, J19943K2)
- Caution: PFA is a known mutagen and irritant and should be handled with care. Collect all PFA containing solutions for disposal according to institutional guidelines.
- Fixation/Permeabilization Solution Kit (BD Biosciences, 554714)
- Perm/Wash Buffer (BD Perm/Wash™, 554723)
- Pe/Cy7 CD49D antibody (BioLegend, 304314)
- Anti- TUJ1 Antibody (Mouse) (BioLegend, 801202)
- Anti-Serotonin-5-HT Antibody (Rabbit) (Sigma, S5545)
- Anti-GABA Antibody (Rabbit) ((Sigma, S5545)
- Anti-NOS1 Antibody (Rabbit) (Santa Cruz Biotechnology, sc648)
- Alexa Fluor 488 donkey anti-mouse IgG (Life Technologies, A21202)
- Alexa Fluor 647 donkey anti-rabbit IgG (Life Technologies, A31573)
- DAPI (Sigma, D9542)
- Caution: DAPI is a known mutagen and should be handled with care. Collect all DAPI containing solutions for disposal according to institutional guidelines.

Gene expression analysis

For qRT-PCR, total RNA was isolated from three biological replicates (~380,000 cells/sample) per time-point using the RNeasy Mini Kit (Qiagen, 74106). Total RNA samples were reverse transcribed to cDNA using SuperScript™ III Reverse Transcriptase (Fisher, 18080044) via the manufacturers protocol. qRT–PCR reactions were then completed with two technical replicates for each of three independent biological replicates per sample using SYBR™ Green PCR Master Mix (Fisher, 4312704) and the 7900HT Fast Real-Time PCR System (Fisher, 4329001). Log2-fold changes in expression were calculated based on the CT value of housekeeping gene GAPDH and CT value of the relevant transcript in hPSCs (before day 0 of differentiation). A list of the qPCR primers used is provided in Supplementary Table 1.

Flow cytometry and immunofluorescence staining

For immunofluorescence, the cells were fixed in 4% PFA in PBS (Alfa Aesar, J19943K2) for 20 min, and then blocked and permeabilized by Perm/Wash Buffer (BD Perm/Wash, 554723). The cells were then incubated in primary antibody overnight at 4 °C, washed with Perm/Wash buffer 3 times, and stained with fluorophore-conjugated secondary antibodies at room temperature for 1 hour. Stained cells were then incubated with DAPI (1Staine^{-S}, Sigma, D9542) and washed an additional 3 times before imaging. For flow cytometry analysis, the cells were dissociated with Accutase (Innovative Cell Technologies, AT104), before being fixed and permeabilized with Fixation/Permeabilization Solution Kit (BD Biosciences, 554714), according to manufacturer's instructions. Single cell suspensions we incubated in primary antibody overnight at 4 °C, washed with BD Perm/Wash™ Buffer 3 times, and stained with fluorophore-conjugated secondary antibodies at room temperature for 1 hour. Flow cytometry was conducted using a BD LSRFortessa. Data was subsequently analyzed with Flowjo software. A list of antibodies and working dilutions is provided in Supplementary Table 2.