SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS AND MATERIALS

Maintenance of hPSCs

Undifferentiated hPSC colonies were propagated using irradiated mouse embryonic fibroblast feeder cells (MEF, Global Stem; 2.5-3 x 10^4 cells/cm^2) in hPSC medium, containing DMEM/F12 with 20% Knockout Serum Replacement, 2 mM L-Glutamine, Pen/Strep (50 U/ml/ 50 µg/ml, respectively), 2 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, and 4 ng/ml bFGF (all from Invitrogen). Collagenase Type IV (Invitrogen) was used to passage the colonies every 5 days at a 1:3 split ratio.

BrdU labeling

Following BrdU (BD Biosciences) incorporation, neocortical organoids were fixed and cryoprotected as described in the main text. The organoids were then exposed to 95% methanol for 10 min, permeabilized with 2 N HCl for 10 min, and neutralized twice with 0.1 M sodium borate for 5 min each. Tissue sections were then blocked, as per the immunocytochemistry protocol described in Materials and Methods, and stained using rabbit anti-BrdU (1:200; 600-401-C29 from Rockland) as the primary antibody and Alexa-Fluor 488 (green) or Alexa-Fluor 555 (red; R&D Systems) as the secondary antibody.

CLARITY of neocortical organoids

The organoids were cleared using the CLARITY protocol modified by using the passive clearing method (Chung *et al*, 2013; Tomer *et al*, 2014).

Clearing: The organoids were picked up using tweezers, dipped into ice cold PBS once, and immediately transferred to 500 µl ice cold Hydrogel Monomer Solution in a 2 ml Conical Screw Cap Tube for 48 h at 4°C. The Hydrogel Monomer Solution was prepared according to previously mentioned protocols (Chung *et al*, 2013; Tomer *et al*,

2014), but without bisacrylamide to prevent the solidifying of the polymerized Hydrogel, which would make removal of the organoids from the hydrogel solution impossible. After 48 h, the tubes were placed in a desiccation chamber, the lids of the tubes were slightly opened and the inlet of the desiccation chamber was filled with nitrogen gas. By turning on a vacuum for 10 min, followed by filling the chamber with nitrogen gas, we were able to replace the gas in the tubes with nitrogen. Afterwards, the sample tubes were quickly closed and incubated at 37°C for 3 h. In a fume hood, the embedded organoids were extracted from the viscous Hydrogel by carefully pipetting them with a pipette tip just wide enough to allow the organoids to enter the pipette tip, while trying to transfer as little excess hydrogel as possible. The organoids were transferred to 500 µl of the clearing solution (Chung *et al*, 2013; Tomer *et al*, 2014), in which the organoids were washed for 24 h at room temperature on a rotator. The organoids were washed two more times with 500 µl clearing solution for 48 h each time at 37°C, followed by passive clearance in clearing solution for 1-2 weeks at 37°C, until they became transparent. During the 1-2 weeks the clearing solution was changed periodically. After the organoids had finished clearing, the organoids were washed twice with 500 µl PBST for 24 h each at room temperature on a rotator.

Immunohistochemistry: Organoids were incubated in 200 µl primary antibody solution (TUJ1 1:1000 in 0.2% triton X-100, 5% goat serum, and 5% BSA in PBS) for 48 h, washed twice in PBST for 24 h each, incubated in secondary antibody solution (1:200 in 0.2% triton X-100 and 2% BSA in PBS) for 48 h, and washed in PBST for 24 h. In order to continue to visualize the organoids when handling them, since they became very difficult to see once cleared, the cleared organoids were stained with DAPI (1:10000 in PBS) for a maximum of 1 h and were washed again for 24 h in PBST. During each step, samples were incubated at room temperature on a rotator.

For refractive indices matching, the organoids were incubated in 300-400 µl 80% glycerol solution for 24 h before imaging. The samples were then embedded in 1% low

melting point agarose gel by pipetting, with the help of a UV light, to visualize the DAPI staining, from the glycerol solution into warm (<40°C) agarose gel in a 60 mm petri dish. After polymerization at room temperature, the embedded organoids were ready for imaging. Images were captured using a Zeiss LSM 710 confocal microscope with ZEN software.

Electrophysiology

Neocortical organoids suspended by means of a suction pipette (ca. 50 µm i.d.) were recorded at 22º C in a bathing medium formulated to match the Neurobasal growth medium. The composition was (mM): NaCl, 83; KCl, 5.3; $CaCl_2$, 1.8; $MgCl_2$, 0.81; HEPES (hemi-sodium), 11.0; glucose, 25; pH 7.4; 220 mOsm. The cells were recorded using an Axopatch 200 B amplifier with capacitance and access resistance compensation and using conventional patch pipettes filled with an intracellular saline composed of (mM): $KMeSO_4$, 88; $MgCl_2$, 2; $CaCl_2$, 1; HEPES-KOH, 10; EGTA, 11; pH 7.2; 210 mOsm.

RT-PCR and RT-qPCR

Total RNA was extracted from hPSCs at different stages of neocortical differentiation using RNA STAT-60 (Tel-Test) and subsequently treated with TURBO DNase (Ambion) to remove residual genomic DNA (Lee *et al*, 2015b; Lee *et al*, 2009). RT-PCR and RT-qPCR were employed to analyze and quantify gene expression, respectively, using cDNA synthesized from DNase-treated RNA (Transcriptor First Strand cDNA Synthesis Kit; Roche).

RT-PCR: RT-PCR was performed using the QIAGEN Multiplex PCR Kit (QIAGEN) according to manufacturer's instructions. Primers for CYP3A isoforms were based on published literatures (Williams *et al*, 2004). Negative controls were performed by omitting cDNA samples, primers, or polymerase during RT-PCR. Primer sequences

and product lengths are listed in Supplementary Table S2.

RT-qPCR: RT-qPCR was performed and analyzed with the LightCycler 480 Real-Time PCR System (Roche) using LightCycler 480 probes Master as described previously (Kindberg *et al*, 2014). The primers, probes, and reference genes (Supplementary Table S2) were designed using Universal ProbeLibrary Assay Design Center (Roche) and their specificities were confirmed by standard and melting curve validation. Measurements were performed in duplicate in two separate runs of 3 independent biological samples at each data point, and all results were normalized to a reference gene, which did not differ between samples. Quantification was performed using the comparative CT method. RT-qPCR of *CYP3A5* and *CYP3A43* was accomplished using the LightCycler® 480 SYBR Green I Master with LightCycler 480 Real-Time PCR System (Roche) following the manufacturer's instructions. Measurements were performed in duplicate in two separate runs of 3-5 independent biological samples, and all results were normalized to GAPDH, which did not differ between samples.

Drugs

Cocaine hydrochloride was provided by the National Institute on Drug Abuse.

Analysis of endogenous ROS formation

ROS content of the neocortical organoids at day 44 was measured by incubating with 100 µM 2', 7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich) for 30 min., either with or without exposure to cocaine. The organoids were washed, then dissolved in 1% Triton X-100 in PBS. Fluorescence was measured at an excitation wavelength of 485 nm, and an emission wavelength of 530 nm using a Techan Genios fluorescence microplate reader. Protein concentrations were determined using the BCA assay (Pierce BCA® Protein Assay Kits; Thermo Scientific), according to the manufacturer's instructions. The level of endogenous ROS was determined by dividing the fluorescence

units by the concentration of protein in the lysate for each sample.

Construction, Packaging, Titering, and Transduction of Lentiviral Vectors

***Knockdown vectors*:** Plasmid shRNA kits for CYP3A5 (Origene# TG313588) and CYP3A43 (Origene# TG305123), containing four different shRNAs for each gene, were tested. shRNA efficiency was examined for gene expression by transient transfection into HEK293 cells. The U6 promoter-shRNA cassettes that gave maximal knockdown for CYP3A5 (TG313588B- AACTGCATTGGCATGAGGTTTGCTCTCAT) and CYP3A43 (TG305123B- GTACTGGACAGAGCCTGAGAAGTTCTGCC), as well as the coding region for eGFP, were amplified by PCR and recombined using In-Fusion cloning mix (Clontech) into pLenti6.3/V5-DEST (Invitrogen) that had been digested with BamHI and MluI restriction enzymes. A control vector was similarly produced using the U6 promoter and a scrambled shRNA (Origene #TR30013). The resulting three vectors, pLenti6.3 CMV eGFP U6 shRNA (CYP3A5, Addgene #61266), pLenti6.3 CMV eGFP U6 shRNA (CYP3A43, Addgene #61265), and pLenti6.3 CMV eGFP U6 shRNA (nonspecific, Addgene #50951), were sequence verified and used to produce lentiviral particles. All lentiviral packaging plasmids were grown in the Stbl3 strain of E. coli (Invitrogen) to minimize recombination of the long terminal repeats.

***Production and Titering of Lentivirus*:** All lentiviral production and experimentation was conducted using Biosafety Level 2 procedures. Lentiviral vectors were packaged according to manufacturer's instructions using the Virapower Packaging system (Invitrogen) which produces replication-defective, VSVG pseudotyped, HIV-1 based lentiviral vector particles. Viral particles were purified and concentrated as described (Lee *et al*, 2015b). Briefly, on the third day after transfection of packaging plasmids into 293FT cells, 20 ml of media was cleared by centrifugation for 5 min x 1000 rpm followed by filtration through a 0.45 µm filter. The filtrate was centrifuged (22,000 rpm in Beckman 28S rotor, 4˚C for 2 h) through 2 ml of 20% sucrose cushion. The supernatant

was aspirated and the viral pellet resuspended in 150 µl of cold HBSS over a 1 h period. Viral particles were aliquoted and frozen at -80˚C. Titering of viral particles was performed using the Lenti-X p24 Rapid Titer Kit (Clontech) according to the manufacturer's instructions. Titers were measured as ng p24/ml and converted to infectious units per ml (IFU) as described in the manual for the Lenti-X p24 Rapid Titer Kit using 500 LP/IFU.

Lentiviral Transduction: Neocortical organoids were plated in either 24-well or 96-well plates for viral infection. Cells were infected by adding 10 µl to each 24-well plate or 2.5 µl to each 96-well plate with a preparation containing $5x10^6$ IFU/ml viral particles. Viral particles were added once to each preparation, at day 28, 4 days prior to the cocaine treatment, and removed at the next change of medium.

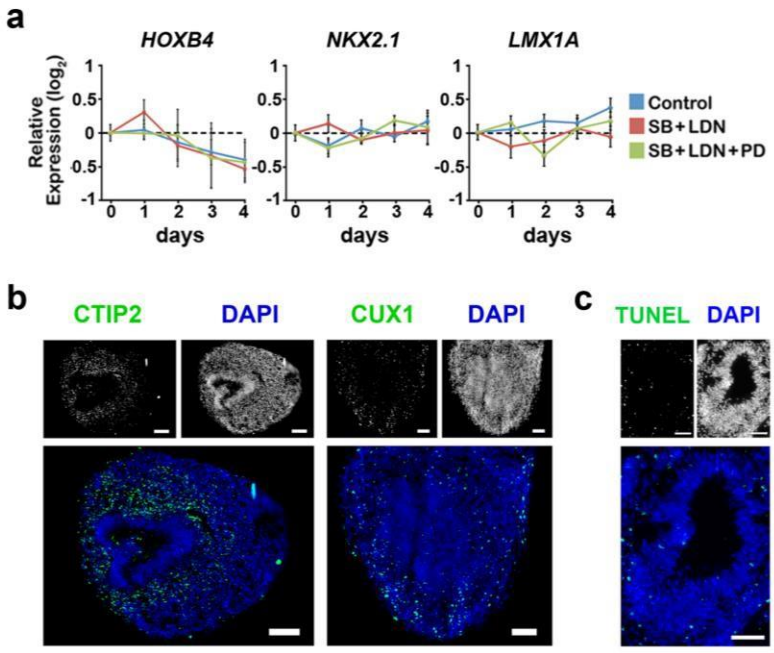
REFERENCES

Lee CT, Lehrmann E, Hayashi T, Amable R, Tsai SY, Chen J *et al* (2009). Gene expression profiling reveals distinct cocaine-responsive genes in human fetal CNS cell types. *J Addict Med* **3**: 218-226.

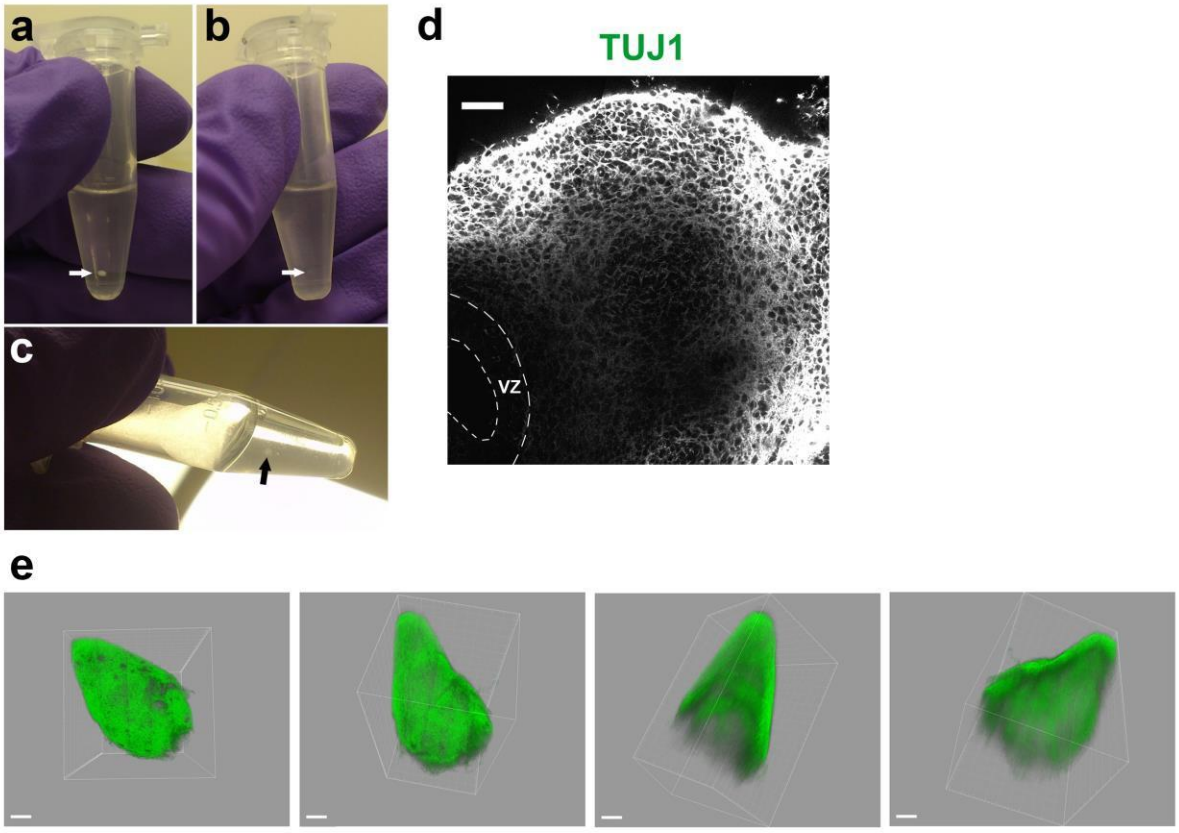
Tomer R, Ye L, Hsueh B, Deisseroth K (2014). Advanced CLARITY for rapid and high-resolution imaging of intact tissues. *Nat Protoc* **9**:1682-1697.

Williams ET, Leyk M, Wrighton SA, Davies PJ, Loose DS, Shipley GL *et al* (2004). Estrogen regulation of the cytochrome P450 3A subfamily in humans. *J Pharmacol Exp Ther* **311**: 728-735.

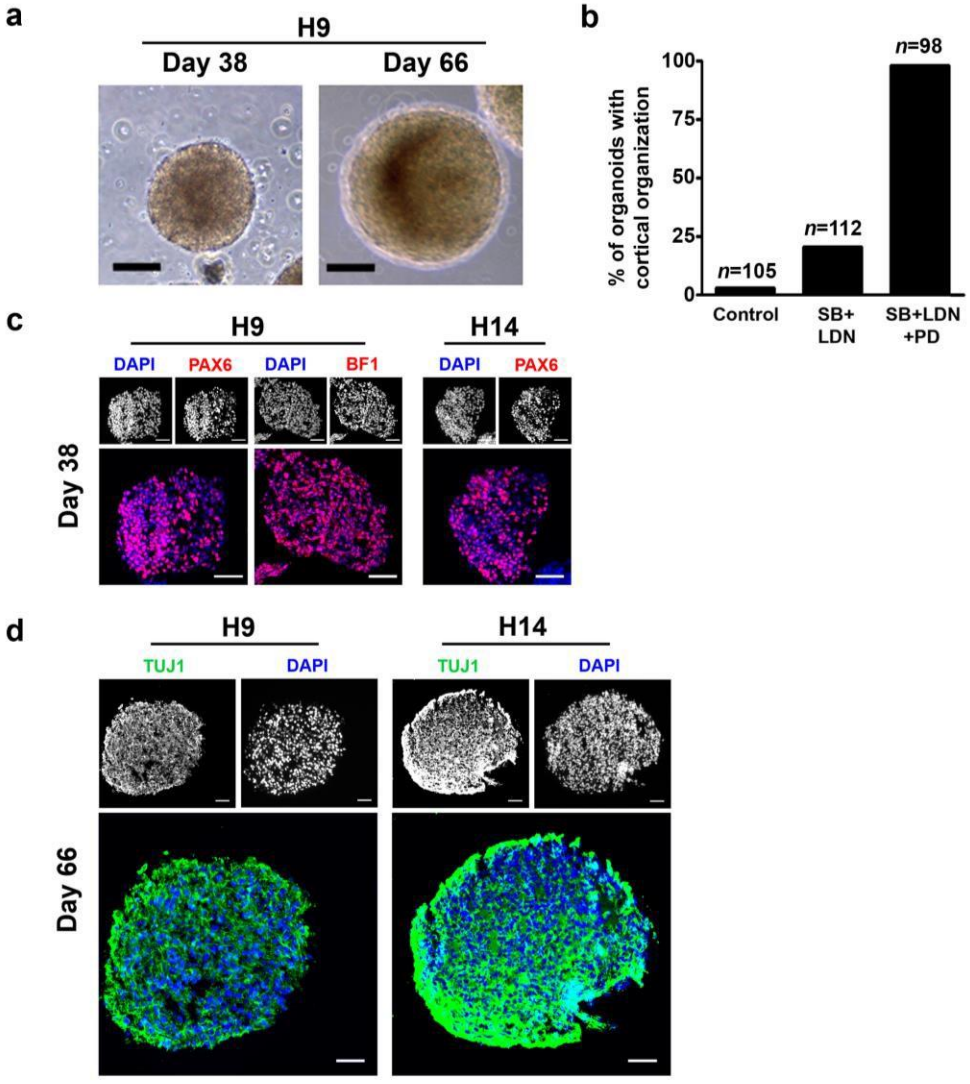
SUPPLEMENTARY FIGURES



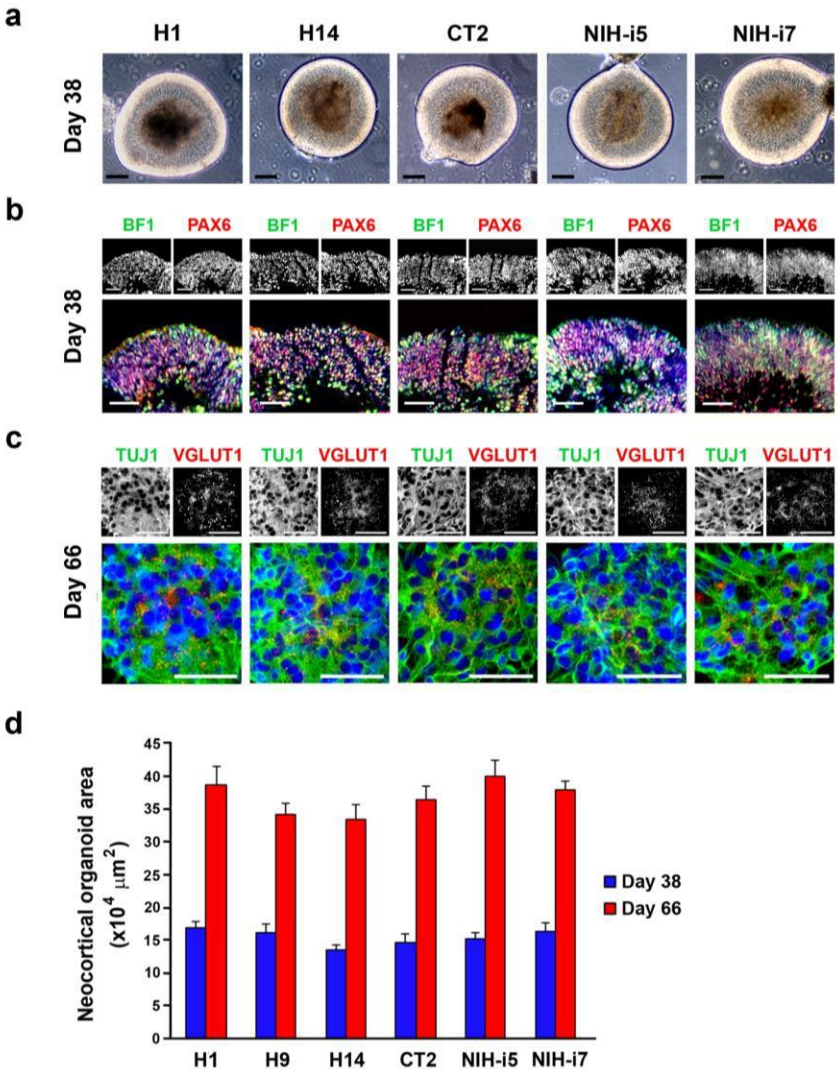
Supplementary Figure S1. Characterization of neocortical organoid differentiation with Dual-SMAD and FGF inhibition. (a) Time-course analysis of gene expressions for neural markers. *n* = 3. Data are shown as means ± s.e.m. (b) Cryosections of neocortical structures immunostained for cortical markers CTIP2 or CUX1 at day 66. Scale bar, 100 µm for CTIP2/DAPI and 50 µm for CUX1/DAPI. (c) TUNEL staining (green) in the interior neuroepithelial region of the neocortical organoids at day 66. Scale bar, 50 µm. Cell line: H9.



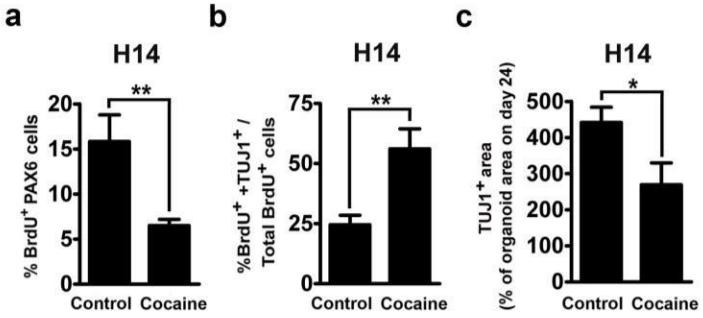
Supplementary Figure S2. Imaging of neocortical organoids from hPSCs using CLARITY. (a-c) Passively cleared neocortical organoid at day 66, using paraformaldehyde-fixed /hydrogel embedded /non-ETC (passive-clearing) methodology, before (a) and after (b, c) clearing (neocortical organoids indicated by arrows). (d) An optical section from near the center of a neocortical organoid at day 66, from an intact organoid immunostained for TUJ1. Scale bar, 50 µm. (e) 3D immunohistological visualization of the intact neocortical organoid at day 66 stained for TUJ1 (green). Scale bar, 100 µm. Cell line: H9.



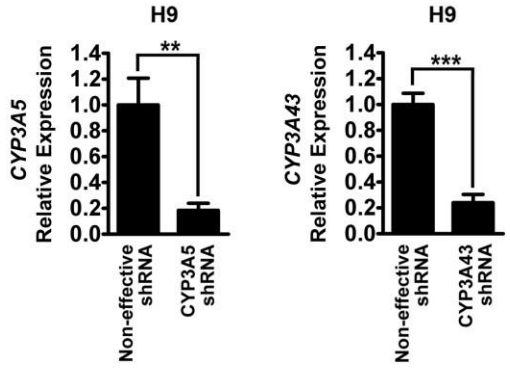
Supplementary Figure S3. Characterization of organoids differentiated from hPSCs with or without inhibitors. (a) Phase-contrast images of floating organoids derived from hPSCs without inhibitors at day 38 and day 66. Scale bar, 100 µm. (b) Analysis of percentages of organoids displaying cortical organization at day 38 using phase contrast microscopy. Cell line: H9. (c, d) Cryosections of organoid structures derived from hPSCs without inhibitors immunostained for various cortical markers, (c) DAPI/PAX6 and DAPI/BF1 at day 38, and (d) TUJ1/DAPI at day 66. Scale bar, 50 µm.



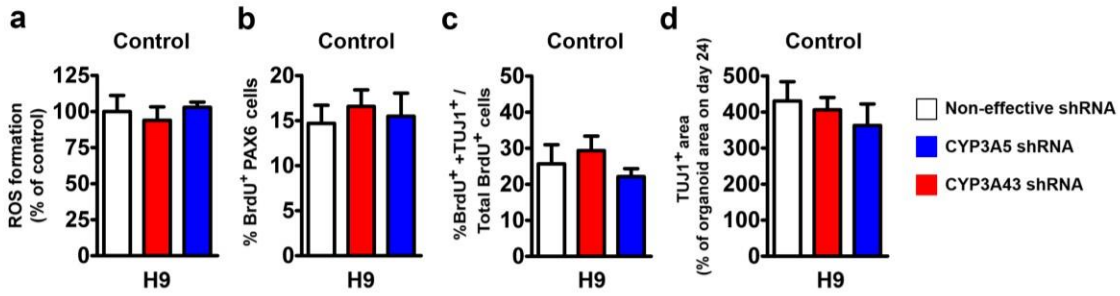
Supplementary Figure S4. Generation of 3D neocortical organoids from multiple hPSC lines by combined dual SMAD and FGF inhibition. (a) Phase-contrast images of floating neocortical organoids derived from various hPSC lines at day 38. Scale bar, 100 µm. (b) Cryosections of neocortical structures immunostained for BF1/PAX6 at day 38. Scale bar, 50 µm. (c) Immunofluorescence staining for TUJ1/VGLUT1 in the neocortical organoids at day 66. Scale bar, 50 µm. (d) Analysis of neocortical organoid areas in various hPSC lines at day 38 and day 66.



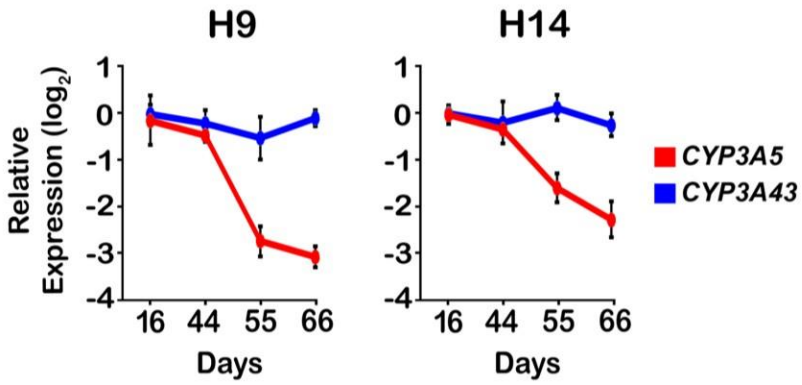
Supplementary Figure S5. Effects of cocaine on the development of neocortical organoids. (a-c) Neocortical organoids were treated with 3 µM cocaine for 1 h every other day from day 32 to day 44. (a) Proliferation of $PAX6^+$ NE cells of neocortical organoids in the presence or absence of cocaine in H14. Proliferation was examined at day 45 with 1 h BrdU (10 µM) incorporation. *n* = 6. (b) Neuronal differentiation in neocortical organoids in the presence or absence of cocaine in H14. Neuronal differentiation was analyzed 24 h after 1 h BrdU (10 µM) treatment from day 51 to day 52. *n* = 6. (c) Neural tissue development in the presence or absence of cocaine in H14. $TUJ1^+$ areas in the neocortical organoids were analyzed at day 66. Total $TUJ1^+$ areas are expressed as percentages of total organoid area on day 24. *n* = 5. Data are shown as means ± s.e.m. Mann-Whitney U-test for a. Unpaired two-tailed Student's t-test for b and c. $*P < 0.05$ and $**P < 0.01$.



Supplementary Figure S6. Lentivirus-mediated CYP3A5 or CYP3A43 knockdown in H9. Lentiviral vectors were employed to silence CYP3A5 or CYP3A43 through delivery of shRNAs at day 28, and relative expression of *CYP3A5* and *CYP3A43* was determined at day 32. $n = 5$. Data are shown as means ± s.e.m. Unpaired two-tailed Student's t-test. $^{**}P < 0.01$ and $^{***}P < 0.001$.



Supplementary Figure S7. Effects of CYP3A5 or CYP3A43 knockdown on the development of neocortical organoids. (a) ROS formation in neocortical organoids (H9) at day 44. *n* = 6. (b) Proliferation of $PAX6^+$ NE cells of neocortical organoids (H9) at day 45 with 1 h BrdU (10 µM) incorporation. *n* = 6. (c) Neuronal differentiation in neocortical organoids (H9). Neuronal differentiation was analyzed 24 h after 1 h BrdU (10 µM) treatment from day 51 to day 52. *n* = 6. (d) Neural tissue development (H9) at day 66. *n* = 6. Data are shown as means ± s.e.m.



Supplementary Figure S8. Time-course analysis of gene expression for *CYP3A5* and *CYP3A43* at different stages of neocortical organoid differentiation in H9 and H14. *n* = 3. Data are shown as means ± s.e.m.

Supplementary Table S1. List of primary antibodies used to characterize the neocortical organoids derived from hPSCs.

Name	Dilution	Catalog #	Brand
BF1	1:100	ab18259	Abcam
CD133	1:100	MAB4310	Millipore
CTIP2	1:50	ab18465	Abcam
CUX1	1:100	sc-13024	Santa Cruz Biotechnology
EMX1	1:50	HPA006421	Sigma
GABA	1:1000	A2052	Sigma
gamma-tubulin	1:500	T6557	Sigma
GFAP	1:1000	Z0334	DAKO
Glutamate	1:2000	G6642	Sigma
HOXB4	1:50	I12 anti-Hoxb4	DSHB
Ki67	1:100	550609	BD Biosciences
N-cadherin	1:100	ab18203	Abcam
Nestin	1:50	MAB1259	R&D Systems
NKX2.1	1:200	MAB5460	Millipore
NEUN	1:500	MAB377	Millipore
PAX6	1:50	PAX6	DSHB
PAX6	1:300	PRB-278P	Covance
PH3	1:500	9701	Cell Signaling Technology
Reelin	1:200	MAB5364	Millipore
TBR1	1:1000	ab31940	Abcam
TUJ1	1:2000	G712A	Promega
VGAT	1:500	131 003	Synaptic Systems
VGluT1	1:500	135 303	Synaptic Systems
VGluT2	1:500	135 403	Synaptic Systems

Supplementary Table S2. RT-PCR or RT-qPCR primers of target genes

RT-PCR				
Gene Name	**Accession #**		**Primer Sequence**	**Fragment Size**
CYP3A4	NM_017460.5	F	tgtttccaagagaagttacaaattttt	91bp
		R	tccactcggtgcttttgtgt	
*CYP3A5**	NM_000777.4	F	actgagtcccacaaagctctgt	71bp
		R	tagccagcaaaaatgaagattattg	
CYP3A7	NM_000765.4	F	ggcccacacctctgcc	66bp
		R	tgtcaaacgtccaatagccc	
*CYP3A43**	NM_022820.4	F	tgctctcacaaacataaaacttgct	85bp
		R	cagtgggatctgagtctctttacaa	
*GAPDH**	NM_001289746.1	F	accacagtccatgccatcac	452bp
		R	tccaccaccctgttgctgta	

*Primers were also employed for RT-qPCR using the LightCycler® 480 SYBR Green I Master with LightCycler 480 Real-Time PCR System.

RT-qPCR					
Gene name	**Accession #**		**Primer sequence**	**Probe #**	**Internal Control**
NANOG	NM_024865.2	F	ccccagcctttactcttcct	Probe 48	TBP
		R	gagaaggcgaaatccgaag		
OCT4	NM_002701.4	F	cttcgcaagccctcatttc	Probe 60	TBP
		R	gagaaggcgaaatccgaag		
T	NM_001270484.1	F	acagcgcatgatcaccag	Probe 38	GAPD
		R	tttgcaaatggattgtacttaatttt		
SOX17	NM_022454.3	F	cgccgagttgagcaagat	Probe 13	GAPD
		R	ggtggtcctgcatgtgct		
Nestin	NM_006617.1	F	tgcgggctactgaaaagttc	Probe 76	GAPD
		R	tgtaggccctgtttctcctg		
OTX2	NM_021728.3	F	aacctcccatgaggctgtaa	Probe 86	GAPD
		R	ggtggacaggttcagagtcc		
PAX6	NM_000280.4	F	ggcacacacacattaacacactt	Probe 9	GAPD
		R	ggtgtgtgagagcaattctcag		
HOXB4	NM_024015.4	F	ctggatgcgcaaagttcac	Probe 62	GAPD
		R	agcggttgtagtgaaattcctt		
NKX2.1	NM_001079668.2	F	tcatttgttggcgactgg	Probe 16	GAPD
		R	tgctttggactcatcgacat		
LMX1A	NM_001174069.1	F	agcattcaaggcctcatttg	Probe 71	GAPD
		R	gacgacacggacactcagc		