SUPPLEMENTAL INFORMATION

Movie S1, Related to Figure 4. 3-D Reconstruction of Confocal Imaging of Neonatal Mouse Ventricular Myocytes Associated with hPSC-Derived Sympathetic Neurons
Immunofluorescence analysis was performed after 7 days following co-culture, using PRPH (pseudo-green, hESC-derived PHOX2B::eGFP+ sympathetic neurons) and cTnT (red, neonatal mouse cardiomyocytes) antibodies.

Movie S2, Related to Figure 4. Propagation of Action Potentials Before and After Neuronal Stimulation
High resolution optical mappings (2 mm × 2 mm, 2 Hz pacing) of neonatal mouse ventricular myocytes co-cultured with hESC-derived sympathetic neurons using voltage-sensitive dye di-4-ANEPPS, showing propagation of action potentials before and after application of 1 μM nicotine.

Movie S3, Related to Figure 4. Spontaneous Beating Synchronization Before and After Neuronal Stimulation
Color-mapped videos of spontaneous beating motions were detected using motion vector analysis of neonatal mouse ventricular myocytes co-cultured with hESC-derived sympathetic neurons, before and 6 min after administration of 1 μM nicotine.

Movie S4, Related to Figure 5. Optogenetic Control on Beating Rates of Neonatal Mouse Ventricular Myocyte Syncytia
Photoactivation of hChR2 in hESC-derived sympathetic neurons leads the increased beating rate of co-cultured neonatal mouse ventricular myocyte syncytia.

Movie S5, Related to Figure 6. Co-Culturing hPSC-Derived Sympathetic Neurons with Neonatal Mouse Ventricular Myocytes Induces the Increased Sensitivity to Nicotine
Calcium imaging analysis of hESC-derived sympathetic neurons with or without neonatal mouse ventricular myocytes after adding 0.1 μM nicotine.

Table S1, Related to all Figures. Details of Culture Condition
Figure number, hPSC-derived neurons or others (source, final cell type, number of days since the pluripotent stage, number of days after lentiviral transduction), myocytes (source, final cell type, number of days after isolation or differentiation), and co-culture (number of days after co-culture).

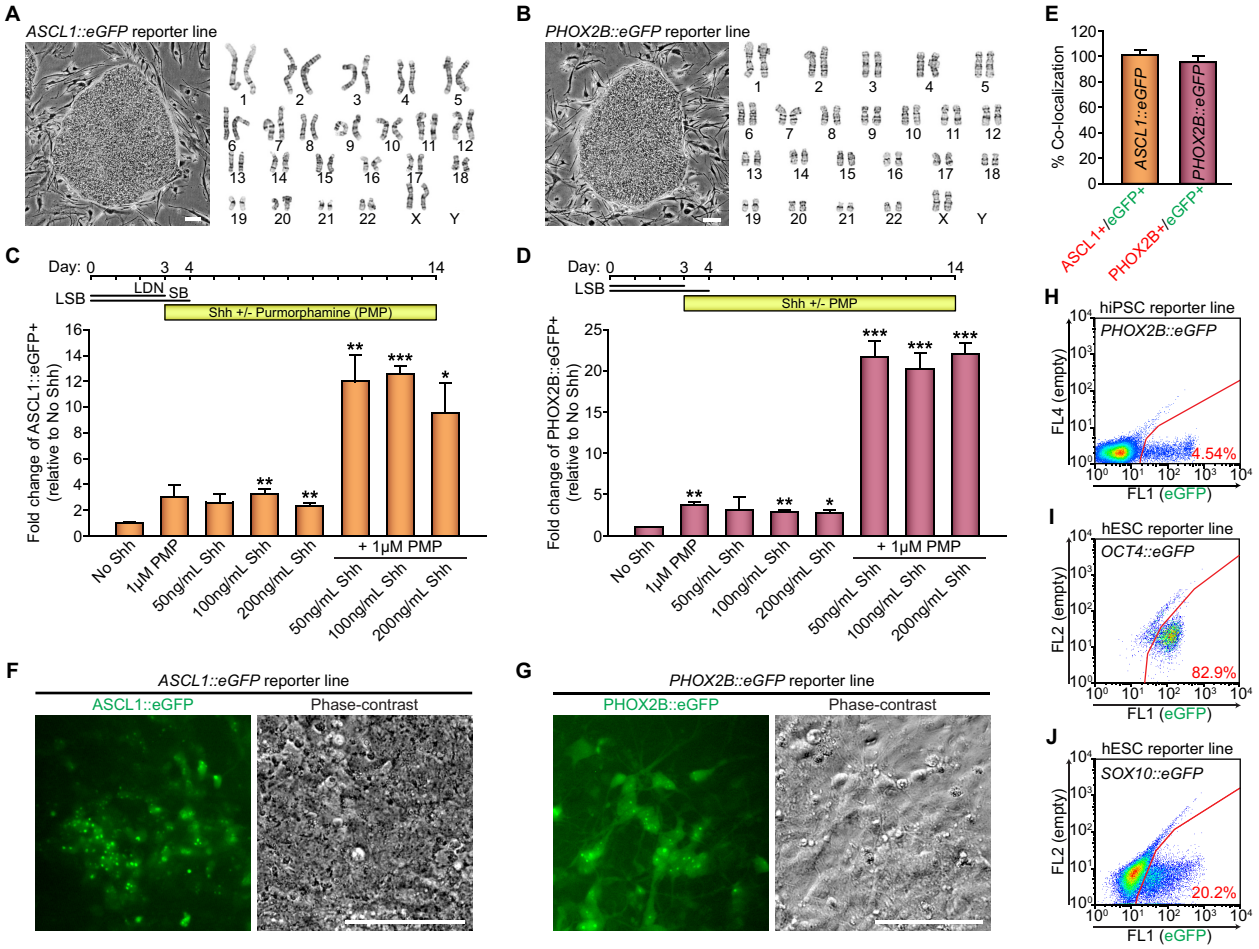

Figure S1, Related to Figure 1. Activation of WNT Followed by SHH Signaling Leads Both ASCL1 and PHOX2B Inductions in hPSC-derived Autonomic Specification

(A-B) Left, representative phase contrast images of *ASCL1::eGFP* (A) and *PHOX2B::eGFP* (B) reporter hESCs; right, cytogenetic G-banding analysis confirmed that both genetic reporter lines using throughout this paper exhibited a normal 46XX karyotype. (C-D) Addition of the recombinant sonic hedgehog C25II (Shh) protein and SHH agonist (purmorphamine, PMP) to the LSB protocol significantly enhanced the production of ASCL1::eGFP- and PHOX2B::eGFP-expressing cells (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; unpaired Student's *t*-test; $n = 3$). LSB, LDN193189 and SB431542. SP, Shh plus PMP. (E) Quantification of the proportion of co-localized cells ($n = 3$). (F-G) Live cell fluorescence and phase-contrast microscopies performed at day 14 following LSB (day 0-3/4) – m3i (day 2-7) – SP (day 3-12) – B4 (day 10-14) treatment. These eGFP signals were too weak to be detected after paraformaldehyde fixation. (H) Representative FACS plots of differentiated reporter hiPSC lines for *PHOX2B::eGFP*. (I-J) Representative FACS plots of (I) *OCT4::eGFP* and (J) *SOX10::eGFP* genetic reporter hESC lines generated by using homologous recombination enhanced by CRISPR/Cas9 system (Cong et al., 2013; Mali et al., 2013). All error bars represent mean + S.E.M. Scale bars, 100 μm.

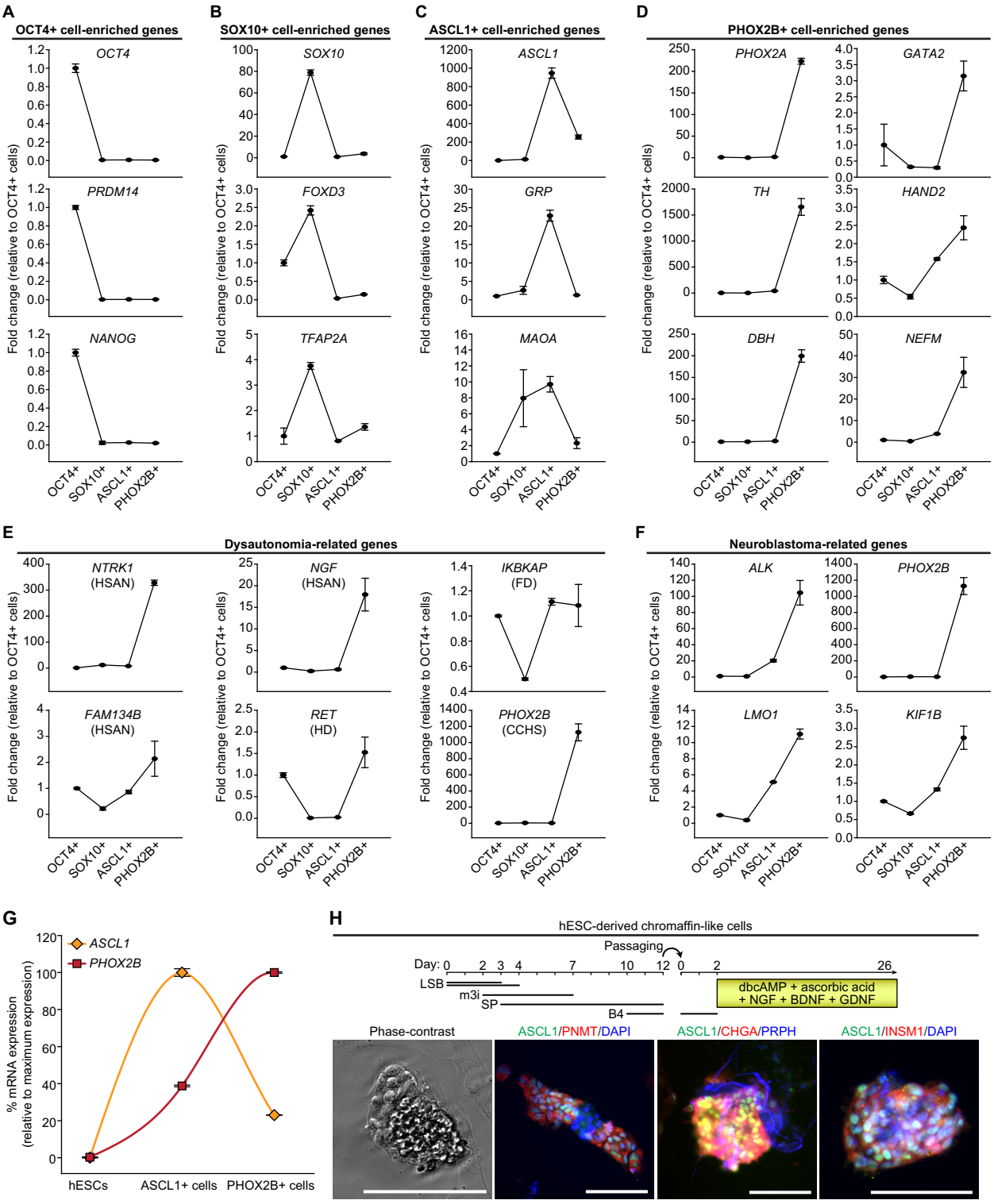

Figure S2, Related to Figure 2. Genetic Reporter hPSC Lines for Four Different Genes Identify Distinct Stages of Sympathetic Neuronal Differentiation

(A-F) Different lines and their specific protocols were as follows: for *OCT4::eGFP*, undifferentiated (day -2); for *SOX10::eGFP* lines, LSB (day 0-3/4) – CHIR (day 2-6.5); for *ASCL1::eGFP* line, LSB (day 0-3/4) – CHIR (day 2-7) – SP (day 3-8); for *PHOX2B::eGFP* line, LSB (day 0-3/4) – m3i (day 2-7) – SP (day 3-10). The eGFP positive

samples were collected by cell sorting at the specific time points (OCT4+ sorting at day -2, SOX10+ sorting at day 6.5, ASCL1+ sorting at day 8, and PHOX2B+ sorting at day 10). qRT-PCR data showing the expression patterns of each transcript during sympathetic neuronal differentiation *in vitro* (n = 3), only except; (C) *GRP*, *MAOA*, and (D) *GATA2*, *NEFM* (these four genes were analyzed from microarray data; n = 3 for OCT4+, SOX10+, or ASCL1+ cells; n = 2 for PHOX2B+ cells). HSAN, hereditary sensory and autonomic neuropathy. HD, Hirschsprung's disease. FD, familial dysautonomia. CCHS, congenital central hypoventilation syndrome. (G) qRT-PCR data showing the expression patterns of *ASCL1* and *PHOX2B* among undifferentiated hESCs, sorted ASCL1+ and PHOX2B+ cells at day 14 following LSB (day 0-3/4) – m3i (day 2-7) – SP (day 3-12) – B4 (day 10-14) treatment (n = 3). (H) Generation of hESC-derived chromaffin-like cells. Left, schematic for generating ASCL1-expressing hESC-derived chromaffin-like cells. Right, Immunofluorescence analyses were performed using ASCL1 (green), PNMT (red), CHGA (red), PRPH (pseudo-blue), and INSM1 (red) antibodies. All error bars represent mean ± S.E.M. Scale bars, 100 μm.

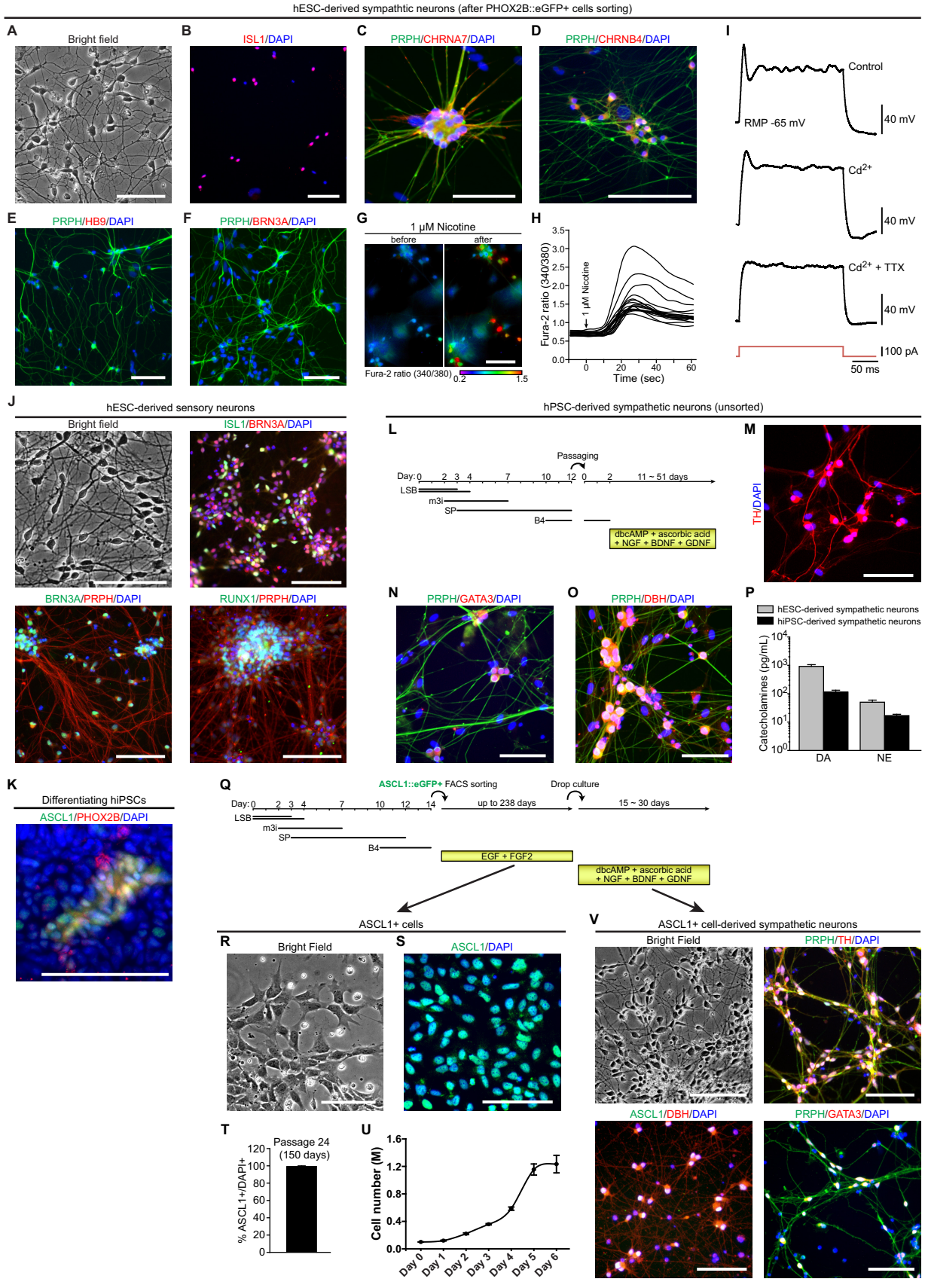
hESC-derived sympathtic neurons (after PHOX2B::eGFP+ cells sorting)
A
Bright field
B
ISL1/DAPI
C
PRPH/CHRNA7/DAPI
D
PRPH/CHRNB4/DAPI
I
Control
RMP -65 mV
40 mV
Cd2+
40 mV
Cd2+ + TTX
40 mV
100 pA
50 ms
E
PRPH/HB9/DAPI
F
PRPH/BRN3A/DAPI
G
1 μM Nicotine
before
after
Fura-2 ratio (340/380)
0.2
1.5
H
Fura-2 ratio (340/380)
1 μM Nicotine
Time (sec)
J
hESC-derived sensory neurons
Bright field
ISL1/BRN3A/DAPI
BRN3A/PRPH/DAPI
RUNX1/PRPH/DAPI
hPSC-derived sympathetic neurons (unsorted)
L
Passaging
Day: 0 2 3 4 7 10 12 0 2 11 ~ 51 days
LSB
m3i
SP
B4
dbcAMP + ascorbic acid
+ NGF + BDNF + GDNF
M
TH/DAPI
N
PRPH/GATA3/DAPI
O
PRPH/DBH/DAPI
P
hESC-derived sympathetic neurons
hiPSC-derived sympathetic neurons
Catecholamines (pg/mL)
DA
NE
K
Differentiating hiPSCs
ASCL1/PHOX2B/DAPI
Q
ASCL1::eGFP+ FACS sorting
Drop culture
Day: 0 2 3 4 7 10 12 14
up to 238 days
15 ~ 30 days
LSB
m3i
SP
B4
EGF + FGF2
dbcAMP + ascorbic acid
+ NGF + BDNF + GDNF
ASCL1+ cells
R
Bright Field
S
ASCL1/DAPI
V
ASCL1+ cell-derived sympathetic neurons
Bright Field
PRPH/TH/DAPI
ASCL1/DBH/DAPI
PRPH/GATA3/DAPI
T
Passage 24
(150 days)
% ASCL1+/DAPI+
U
Cell number (M)
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6

Figure S3, Related to Figure 3. The Characteristics of hPSC-Derived Sympathetic Neurons

(A-I) The characteristics of hESC-derived sympathetic neurons. Following LSB (day 0-3/4) – m3i (day 2-7) – SP (day 3-12) – B4 (day 10-14) treatment, sorted PHOX2B::eGFP+ cells were further differentiated into putative sympathetic neurons in the presence of dbcAMP, ascorbic acid, BDNF, NGF, and GDNF. LSB, LDN193189 and SB431542. m3i, CHIR99021, DAPT, and PD173074. SP, Shh plus PMP (purmorphamine). B4, BMP4. (A) Representative bright field image of hESC-derived sympathetic neurons 4 days after cell sorting. (B-F) Immunofluorescence analyses were performed using ISL1 (red), PRPH (pseudo-green), CHRNA7 (red), CHRNB4 (red), HB9 (red), and BRN3A (red) antibodies. (G) Representative heat map images of the ratio of Fura-2 light emission on excitation at 340 and 380 nm (340/380) in the hESC-derived sympathetic neurons showing changes in intracellular calcium concentrations $[Ca^{2+}]_i$ induced by bath application of 1 μM nicotine. (h) Example traces of $[Ca^{2+}]_i$, measured by ratiometric Fura-2 imaging, from hESC-derived sympathetic neurons exposed to 1 μM nicotine. Each trace is a response from a unique cell (n = 18). (I) The action potentials of these neurons were partially blocked with Cd^{2+} alone and completely blocked with Cd^{2+} + TTX showing its dependency on Ca^{2+} and Na^{+} ions. RMP, resting membrane potential. (J) Representative bright field image and immunofluorescence images of hESC-derived sensory neurons differentiated by using previously published protocol (Chambers et al., 2012). Immunofluorescence analyses were performed using ISL1 (green), BRN3A (red or green), PRPH (red), and RUNX1 (green) antibodies. (K) After LSB (day 0-3/4) – m3i (day 2-7) – SP (day 3-12) – B4 (day 10-14) treatment, an immunofluorescence analysis of differentiating hiPSCs was performed using ASCL1 (green) and PHOX2B (red) antibodies. (L-P) Unsorted hPSC-derived sympathetic neurons. (L) Schematic for generating hPSC-derived sympathetic neurons without using either cell sorting or any reporter lines. (M-O) Immunofluorescence analyses were performed using TH (red), PRPH (green), GATA3 (red), and DBH (red) antibodies. (P) The releases of catecholamines after 50 mM KCl administration were analyzed by using commercial ELISA kit (n = 3). (Q-V) ASCL1+ cells can be cultured for over 150 days without losing ability to differentiate into sympathetic neurons. (Q) Schematic for differentiation of ASCL1::eGFP positive cells. (R-U) After cell sorting, FACS-purified ASCL1::eGFP+ cells were maintained over 150 days. (R) Representative bright field image of hESC-derived ASCL1+ cells 150 days after sorting. Immunofluorescence analyses of these cells were performed using (S) ASCL1 (green) antibody, and (T) ASCL1 positive cells were quantified (n = 3). (U) Growth curve of hESC-derived ASCL1+ cells 150 days after sorting (n = 3). (V) After dropping cells, the cells were further differentiated into ASCL1+ cell-derived sympathetic neurons in the presence of dbcAMP, ascorbic acid, BDNF, NGF, and GDNF. Immunofluorescence analyses were performed using PRPH (pseudo-green), TH (red), ASCL1 (green), DBH (red), and GATA3 (green) antibodies. All error bars represent mean + S.E.M. Scale bars, 100 μm.

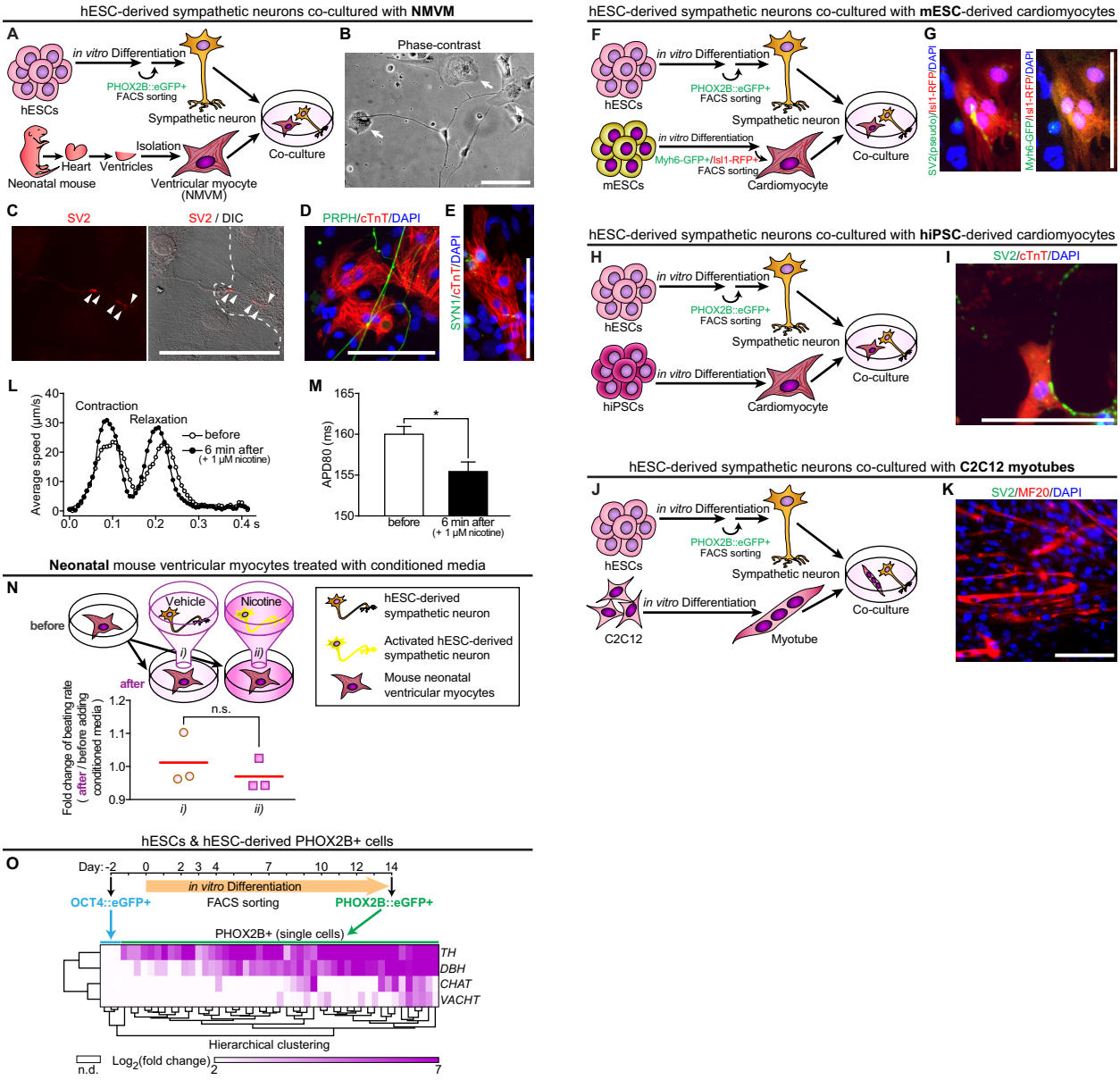

Figure S4, Related to Figure 4. hPSC-Derived Sympathetic Neurons Were Physically Associated with Neonatal Mouse Ventricular Myocytes (NMVM) or mESC/hiPSC-Derived Cardiomyocytes

(A, F, H, and J) Schematic representation of the *in vitro* co-culture system, with FACS-purified PHOX2B::eGFP+ sympathetic neurons and either (A) NMVM, (F) mESC-derived cardiomyocytes, (H) hiPSC-derived cardiomyocytes, or (J) C2C12-derived myotubes, used to test sympathetic connection. (B) Representative bright field image of hESC-derived sympathetic neurons with beating cardiomyocytes. After one day following co-culture, physical contacts of neurites with cardiomyocytes were detected. White arrows indicate NMVM with beating. (C-E, G, and I) Immunofluorescence analyses were performed using SYN1 (pseudo-green), cTnT (red), SV2 (red in C, pseudo-green in G and I), and PRPH (pseudo-green) antibodies. (C) The fluorescence image for SV2 was merged onto bright field image. White arrowheads indicate SV2 positive vesicles. White dashed line indicates the boundary of cardiomyocyte. (G) The co-expression of Myh6-GFP and Isl1-RFP was used as a marker of mESC-derived cardiomyocytes with beating. (K) Immunofluorescence analyses were performed using SV2 (pseudo-green) and MF20 (red) antibodies. (L) Representative motion waveform of NMVM co-cultured with hESC-derived PHOX2B::eGFP+ neurons before and 6 min after administration of 1 μM nicotine. First peak indicates a contraction, and second peak indicates a relaxation.

(M) Action potential duration at 80% repolarization (APD80) for NMVM co-cultured with hESC-derived PHOX2B::eGFP+ neurons, before and 6min after application of 1 μM nicotine. Nicotine caused a slight but significant shortening in APD80 ($P < 0.05$; unpaired Student's *t*-test; $n = 7$). All error bars represent mean + S.E.M. (N) The conditioned media were used to check paracrine effect of hESC-derived sympathetic neurons. After NMVM were fed with the 6 min conditioned media (from either vehicle or 1 μM nicotine-treated human sympathetic neurons) for 6 min, the effect of the conditioned media on the beating rates of NMVM were analyzed (n.s., not significant; unpaired Student's *t*-test; $n = 3$). (O) hESC-derived PHOX2B+ cells (14 days after differentiation from hESC) were collected by single cell sorting, and OCT4+ cells (hESCs) were also purified by cell sorting as a control. Single cell qRT-PCR was performed using indicated primers ($n = 3$ for OCT4+ cells; $n = 47$ for PHOX2B+ single cells). Scale bars, 100 μm.

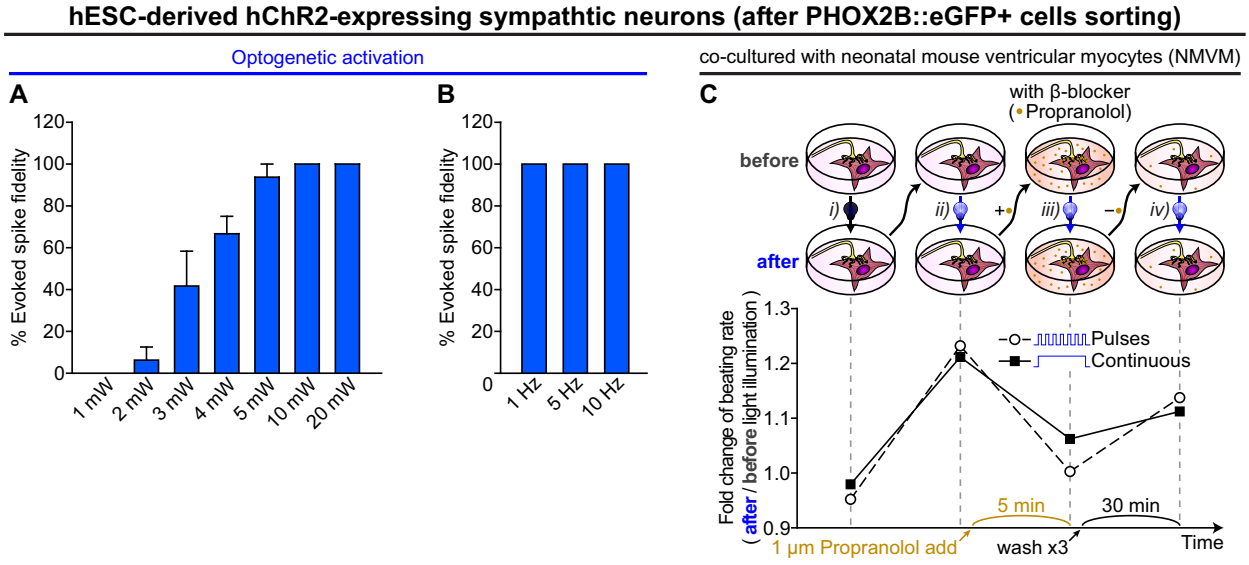

Figure S5, Related to Figure 5. Optogenetic Control on hPSC-derived Sympathetic Neurons Lead to Changes of Spontaneous Beating of Neonatal Mouse Ventricular Myocytes

(A-B) hESC-derived sympathetic neurons were infected with hChR2 lentivirus. (A) Optogenetically-evoked spike fidelity by wide-field blue light (1-20 mW at 470 nm, 5 Hz, 1 ms) plotted as a function of light intensity (n = 4 for 1 mW, 2 mW, 5 mW; n = 3 for 3 mW, 4 mW; n = 9 for 10 mW, 20mW). (B) Evoked spike fidelity by wide-field blue light (20 mW at 470 nm, 1-10 Hz, 1 ms) was constant over a wide range of stimulation frequencies (n = 4 for 1 Hz, n = 9 for 5 Hz, 10 Hz). All error bars represent mean + S.E.M. (C) Photoactivation of hChR2-expressing sympathetic neurons co-cultured with NMVM using continuous focal blue light or pulsed focal blue light. Different from conventional wide-field illumination, the field diaphragm in the epi-illumination pathway of the microscope was fully closed in order to achieve focal illumination (area, 0.08 mm^2). Light intensity was 26 mW/mm^2 at 488 nm. Before and after illumination with pulsed (~4 Hz, ~100 ms) or continuous blue light, spontaneous beating rates were measured in the order of four sections (~20 sec each): *i)* 'no photostimulation', *ii)* 'before propranolol administration', *iii)* '5 min after administration of 1 μM propranolol', and *iv)* '30 min after washout propranolol'.

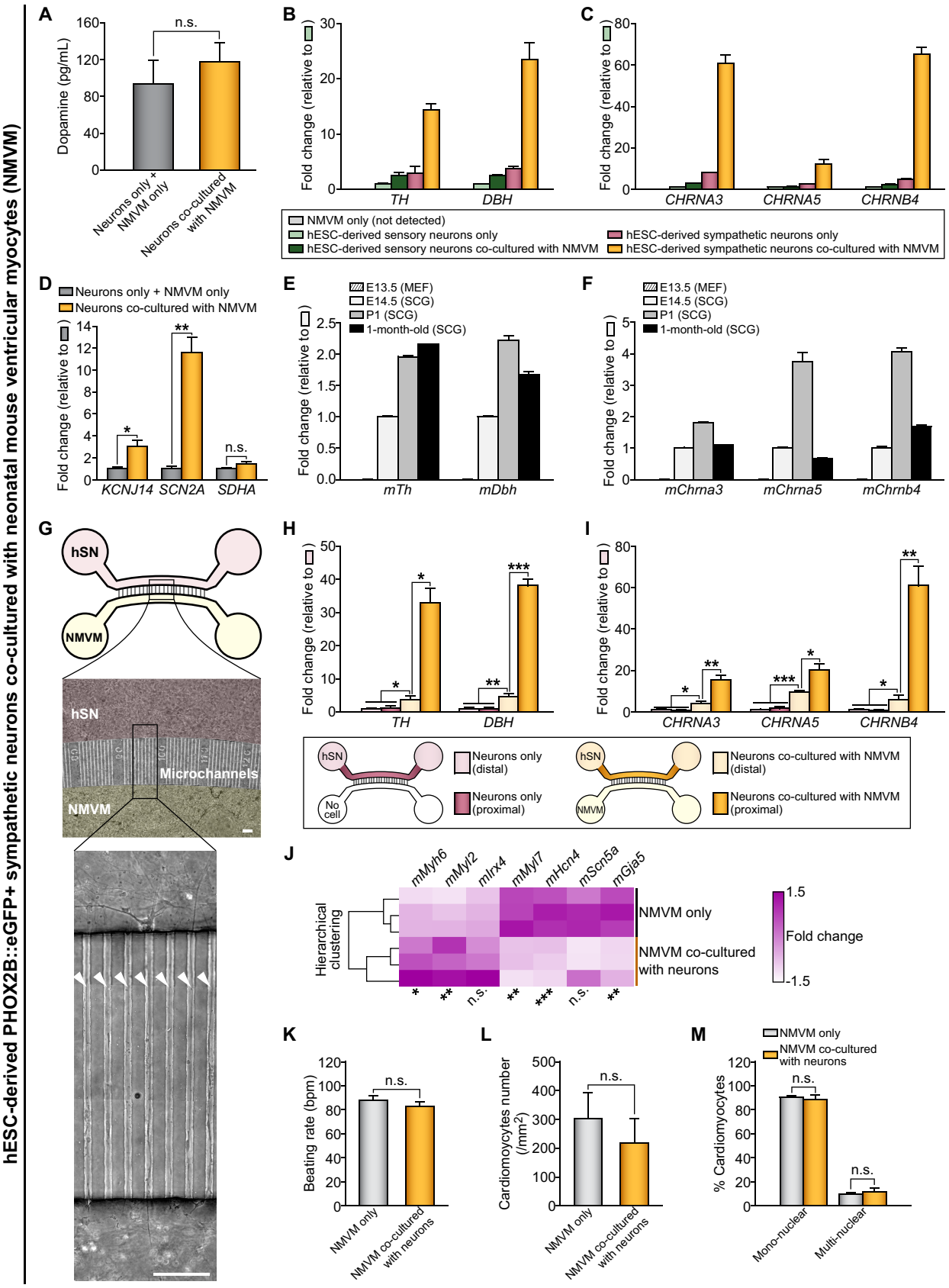
hESC-derived PHOX2B::eGFP+ sympathetic neurons co-cultured with neonatal mouse ventricular myocytes (NMVM)
A
Dopamine (pg/mL)
n.s.
Neurons only + NMVM only
Neurons co-cultured with NMVM
B
Fold change (relative to)
TH
DBH
C
CHRNA3
CHRNA5
CHRNB4
NMVM only (not detected)
hESC-derived sensory neurons only
hESC-derived sensory neurons co-cultured with NMVM
hESC-derived sympathetic neurons only
hESC-derived sympathetic neurons co-cultured with NMVM
D
Neurons only + NMVM only
Neurons co-cultured with NMVM
KCNJ14
SCN2A
SDHA
E
E13.5 (MEF)
E14.5 (SCG)
P1 (SCG)
1-month-old (SCG)
mTh
mDbh
F
mChrna3
mChrna5
mChrnb4
G
hSN
NMVM
Microchannels
H
I
Neurons only (distal)
Neurons only (proximal)
Neurons co-cultured with NMVM (distal)
Neurons co-cultured with NMVM (proximal)
No cell
J
mMyh6
mMyl2
mIrx4
mMyl7
mHcn4
mScn5a
mGja5
Hierarchical clustering
NMVM only
NMVM co-cultured with neurons
Fold change
1.5
-1.5
K
Beating rate (bpm)
L
Cardiomyocytes number (/mm2)
M
% Cardiomyocytes
Mono-nuclear
Multi-nuclear

Figure S6, Related to Figure 6. Co-Culturing hPSC-Derived Sympathetic Neurons with Neonatal Mouse Ventricular Myocytes (NMVM) Leads to the Neuronal Maturation Phenotypes

(A-D) After 14 days following PHOX2B::eGFP+ cells sorting, NMVM or mock ('neurons only') were added onto hESC-derived sympathetic neurons ('neurons with NMVM) or mock ('NMVM only') with 1:1 mixed medium (mouse cardiomyocytes medium : neuron medium; *see* Supplemental Experimental Procedures for more details). (A) After 7 days following co-culture, the amounts of dopamine release after 50 mM KCl administration were analyzed by using commercial ELISA kit (n.s., not significant; unpaired Student's *t*-test; $n = 3$). (B-C) hESC-derived sensory neurons differentiated by using previously published protocol (Chambers et al., 2012) were also co-cultured with NMVM ('hESC-derived sensory neurons with NMVM) or mock ('hESC-derived sensory neurons only'). After 7 days following single or co-culture, qRT-PCR analyses were performed by using indicated human-specific primers ($n = 3$). (D) After 7 days following single or co-culture, total RNA from all samples were extracted. The RNA from 'neurons only' samples and 'cardiomyocytes only' samples were mixed together and used as a 'neurons only + NMVM only' control. Then, qRT-PCR analyses were performed by using indicated human-specific primers (n.s., not significant; $*P < 0.05$; $**P < 0.01$; unpaired Student's *t*-test; $n = 3$). Another housekeeping gene, *SDHA*, was used as a negative control. (E-F) Dissected mouse superior cervical ganglia (SCG) in the indicated time point were used as sympathetic neurons *in vivo* and mouse embryonic fibroblasts (MEFs) were used as a negative control of mouse SCG. qRT-PCR analyses were performed by using indicated mouse-specific primers ($n = 3$). (G) Schematic representation of the *in vitro* co-culture system, with FACS-purified PHOX2B::eGFP+ sympathetic neurons (hSN, human sympathetic neurons) and neonatal mouse ventricular myocytes (NMVM), using the microfluidic chamber device. White arrowheads indicate bunches of the neurites of hSNs growing toward NMVM side. (H-I) qRT-PCR analyses were performed by using indicated human-specific primers ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$; unpaired Student's *t*-test; $n = 3$). Scale bars, 100 μm. (J). qRT-PCR analyses were performed by using indicated mouse-specific primers and shown by the hierarchically clustering heat map (n.s., not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; unpaired Student's *t*-test; $n = 3$). (K) The absolute value of beating rate of NMVM with or without co-cultured human sympathetic neurons. This graph represents whole absolute values of spontaneous beating rate, without neuronal stimulation, used in this paper (n.s., not significant; unpaired Student's *t*-test; $n = 48$ for 'NMVM only'; $n = 86$ for 'NMVM co-cultured with neurons'). (L) The number of cTnT positive cardiomyocytes per mm^2 with or without co-cultured hESC-derived sympathetic neurons (n.s., not significant; unpaired Student's *t*-test; $n = 5$). (M) The percent of cTnT positive mono-nuclear cells and multi-nuclear cells with or without co-cultured hESC-derived sympathetic neurons (n.s., not significant; unpaired Student's *t*-test; $n = 5$). All error bars represent mean + S.E.M.

Table S1, Related to all Figures. Details of Culture Condition

	hPSC-derived neurons or others				Myocytes			Co-culture
Figure No.	Source	Final cell type	Number of days since the pluripotent stage (day 0)	Number of days after lentiviral transduction	Source	Final cell type	Number of days after isolation or differentiation	Number of days after co-culture
1B, 1E	hESC	hESC	0					
1C, 1F, 1G, 1J	hESC	Mixed	14					
1H-1I	hESC	Mixed	14					
1K	hESC	Mixed	10					
1L	hESC	ASCL1±, PHOX2B±	14					
2A-2H	hESC	OCT4+	0					
2A-2H	hESC	SOX10+	6.5					
2A-2H	hESC	ASCL1+	8					
2A-2H	hESC	PHOX2B+	10					
3B (Top-Left)	hESC	Sympathetic neuron	18					
3B (Top-Right)	hESC	Sympathetic neuron	21					
3B (Bottom-Left)	hESC	Sympathetic neuron	21					
3B (Bottom-Right)	hESC	Sympathetic neuron	29					
3C	hESC	Sympathetic neuron	21-29					
3D	hESC	Sympathetic neuron	29					
3E-3H	hESC	Sympathetic neuron	28					
3I	hESC	Sensory neuron	31					
3I	hESC	Sympathetic neuron	34					
4B	hESC	Sympathetic neuron	42		P1 mouse	Ventricular myocyte	7	7
4C	hESC	Sympathetic neuron	29		P1 mouse	Ventricular myocyte	7	7
4D *i)*					P1 mouse	Ventricular myocyte	7-9	
4D *ii)*	hESC	Sensory neuron	29-31		P1 mouse	Ventricular myocyte	7-9	7-9
4D *iii)*	hESC	Sympathetic neuron	28-35		P1 mouse	Ventricular myocyte	7-9	7-9
4D *iv)*	hESC	Sympathetic neuron	28-35		P1 mouse	Ventricular myocyte	7-8	7-8
4E-4F	hESC	Sympathetic neuron	24		P1 mouse	Ventricular myocyte	6	6
5B-5C	hESC	Sympathetic neuron	44	14				
5D, 5F	hESC	Sympathetic neuron	40-44	11-15				
5G *i)*					P1 mouse	Ventricular myocyte	7	
5G *ii)*	hESC	Sympathetic neuron	32-33	10-11	P1 mouse	Ventricular myocyte	7-8	7-8
5G *iii)*	hESC	Sympathetic neuron	25-32	11-14	P1 mouse	Ventricular myocyte	7-8	7-8
5G *iv)*	hESC	Sympathetic neuron	28-29	13-14	P1 mouse	Ventricular myocyte	7-8	7-8
5H	hESC	Sympathetic neuron	28-36	12-22	P1 mouse	Ventricular myocyte	8	8
5I	hESC	Sympathetic neuron	31-42	12-23	P1 mouse	Ventricular myocyte	8	8
6A	hESC	Sympathetic neuron	36		P1 mouse	Ventricular myocyte	7	7 or 0
6B-6C	hESC	Sympathetic neuron	35		P1 mouse	Ventricular myocyte	7	7 or 0
6D-6G	hESC	Sympathetic neuron	36					
6D-6G	hESC	Sympathetic neuron	36		P1 mouse	Ventricular myocyte	8	8
S1A-S1B	hESC	hESC	0					
S1C-S1H	hESC	Mixed	14					
S1I	hESC	hESC	0					
S1J	hESC	Mixed	6.5					
S2A-S2F	hESC	OCT4+	0					
S2A-S2F	hESC	SOX10+	6.5					
S2A-S2F	hESC	ASCL1+	8					
S2A-S2F	hESC	PHOX2B+	10					
S2G	hESC	ASCL1+, PHOX2B+	14					
S2H	hESC	Chromaffin-like	38					
S3A	hESC	Sympathetic neuron	18					
S3B	hESC	Sympathetic neuron	22					
S3C-S3D	hESC	Sympathetic neuron	30					
S3E-S3F	hESC	Sympathetic neuron	22					
S3G-S3H	hESC	Sympathetic neuron	36					
S3I	hESC	Sympathetic neuron	28					
S3J	hESC	Sensory neuron	29-35					
S3K	hESC	Mixed	14					
S3M	hESC	Sympathetic neuron	34					
S3N	hESC	Sympathetic neuron	25					
S3O	hESC	Sympathetic neuron	23					
S3P	hESC	Sympathetic neuron	63					
S3P	hiPSC	Sympathetic neuron	53					
S3R-S3T	hESC	ASCL1+	164					
S3U	hESC	ASCL1+	164-170					
S3V (Top-Left)	hESC	Sympathetic neuron	48					
S3V (Right panels)	hESC	Sympathetic neuron	268					
S3V (Bottom-Left)	hESC	Sympathetic neuron	88					

	hPSC-derived neurons or others				Myocytes			Co-culture
Figure No.	Source	Final cell type	Number of days since the pluripotent stage (day 0)	Number of days after lentiviral transduction	Source	Final cell type	Number of days after isolation or differentiation	Number of days after co-culture
S4B	hESC	Sympathetic neuron	17		P1 mouse	Ventricular myocyte	1	1
S4C	hESC	Sympathetic neuron	32		P1 mouse	Ventricular myocyte	7	7
S4D-S4E	hESC	Sympathetic neuron	29		P1 mouse	Ventricular myocyte	7	7
S4G	hESC	Sympathetic neuron	27		mESC	Cardiomyocyte	18	7
S4I	hESC	Sympathetic neuron	58		hiPSC	Cardiomyocyte	44	30
S4K	hESC	Sympathetic neuron	31		C2C12	Skeletal myotube	5	3
S4L	hESC	Sympathetic neuron	46		P1 mouse	Ventricular myocyte	28	28
S4M	hESC	Sympathetic neuron	34		P1 mouse	Ventricular myocyte	7	7
S4N	hESC	Sympathetic neuron	42		P1 mouse	Ventricular myocyte	7	
S4O	hESC	OCT4+, PHOX2B+	0, 14					
S5A-S5B	hESC	Sympathetic neuron	42	13				
S5C	hESC	Sympathetic neuron	31	12	P1 mouse	Ventricular myocyte	8	8
S6A	hESC	Sympathetic neuron	35		P1 mouse	Ventricular myocyte	7	7 or 0
S6B-S6C					P1 mouse	Ventricular myocyte	7	
S6B-S6C	hESC	Sensory neuron	27		P1 mouse	Ventricular myocyte	7	7 or 0
S6B-S6C	hESC	Sympathetic neuron	35		P1 mouse	Ventricular myocyte	7	7 or 0
S6D	hESC	Sympathetic neuron	35		P1 mouse	Ventricular myocyte	7	7 or 0
S6G-S6I	hESC	Sympathetic neuron	30		P1 mouse	Ventricular myocyte	9	9 or 0
S6J					P1 mouse	Ventricular myocyte	7	
S6J	hESC	Sympathetic neuron	36		P1 mouse	Ventricular myocyte	7	7
S6K					P1 mouse	Ventricular myocyte	7-9	
S6K	hESC	Sympathetic neuron	24-46	0 or 10-23	P1 mouse	Ventricular myocyte	6, 7-9, 28	6, 7-9, 28
S6L					P1 mouse	Ventricular myocyte	7	
S6L	hESC	Sympathetic neuron	35		P1 mouse	Ventricular myocyte	7	7
S6M					P1 mouse	Ventricular myocyte	7	
S6M	hESC	Sympathetic neuron	35		P1 mouse	Ventricular myocyte	7	7

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of Reporter Knock-in hPSC Lines by Homologous Recombination

Feeder-free H9 hESCs or control 01582 iPSCs were dissociated into single cells using Accutase (Innovative Cell Technologies), and 2×10^6 cells were resuspended in nucleofection solution V (Lonza) with 4 μg hCas9 plasmid, 4 μg gRNA, and 4 μg dsDNA donor plasmids for either *ASCL1* or *PHOX2B* (*see* Plasmid Constructions), and nucleofected by Nucleofector™ II according to manufacturer's instruction (using B-16 program, Lonza). The nucleofected cell suspension was subsequently plated on the puromycin-resistant MEFs (DR4, Global Stem) in hESC medium with 10 μM Y-27632 (Cayman Chemical). Four days after nucleofection, cells that had undergone homologous recombination were selected by adding 0.5 μg/ml of puromycin in a medium containing DMEM/F12, 20% knockout serum replacement (KSR), 0.1 mM MEM-NEAA, 1 mM L-glutamine, 55 μM β-mercaptoethanol (Life Technologies) and 4 ng/mL FGF2 (R&D Systems) (hESC medium). After selection, puromycin-resistant colonies were screened for successful knock-in by qPCR of genomic DNA (*2A-eGFP*, forward 5'-GTTGAAACAAGCAGGGGATGTC-3', reverse 5'-TTGTGGCCGTTTACGTCGC-3'). The *OCT4::eGFP* reporter hESC line was generated as previously described (Hockemeyer et al., 2011). To verify the chromosomal aberrations, karyotype analyses were performed on the candidate clones (Cell Line Genetics, Inc.).

Plasmid Constructions

The dsDNA donor vectors for homologous recombination at the naïve *ASCL1* and *PHOX2B* loci are designed to have 2A-eGFP-PGK-Puro gene cassettes between both homology arms, using OCT4-2A-eGFP-PGK-Puro vector (*OCT4* donor vector (Hockemeyer et al., 2011), Addgene plasmid #31938) as a backbone. Each homology arm has 1,817 bp upstream sequences (*ASCL1* left arm) or 915 bp downstream sequences (*ASCL1* right arm) from the stop codon of *ASCL1*, and 913 bp upstream sequences (*PHOX2B* left arm) or 913 bp downstream sequences (*PHOX2B* right arm) from the stop codon of *PHOX2B*. The homology arms in *ASCL1* and *PHOX2B* donor vectors were generated by PCR of human genomic DNA in the WA09 (H9) hESC line (Thomson et al., 1998) and insertion of the PCR-amplified *ASCL1* left homology arm (forward primer: 5'-GCCATA*GAGCTC*AGTGAGGAGAGAGAGAAAACAG-3', reverse primer: 5'-GCCATA*GCTAGC*GAACCAGTTGGTGAAGTCGAG-3') between the *Sac* I and *Nhe* I sites, the *ASCL1* right homology arm (forward primer: 5'-GCCATA*GGCGCGCC*GGGGCTCGGCCTGG-3', reverse primer: 5'-GCCATA*GCGGCCGC*GGGCTGAGAATAGCATAGTTTCAC-3') between the *Asc* I and *Not* I sites, *PHOX2B* left homology arm (forward primer: 5'-GCCATA*GGATCC*CTTTTCCTTCTACCAATTAG-3', reverse primer: 5'-GCCATA*GCTAGC*GAACATACTGCTCTTCACTAAGGC-3') between the *Bam*H I and *Nhe* I sites, and the *PHOX2B* right homology arm (forward primer: 5'-GCCATA*GGCGCGCC*TCTGGAATCCTGCGGCG-3', reverse primer: 5'-GCCATA*GGCCGGCC*GAAAAGGCATCTCATGGATAGGG-3') between the *Asc* I and *Fse* I sites, respectively, in the OCT4-2A-eGFP-PGK-Puro vector. The gRNA target sequences for *ASCL1*, and *PHOX2B* were chosen near the stop codon of each gene and subcloned into gRNA_Cloning Vector (Addgene plasmid #41824) as described previously (Mali et al., 2013). The oligonucleotides for gRNA constructs were as follows: *ASCL1*-gRNA, forward 5'-TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCCCTGGTGCGAATGGACTT-3', reverse 5'-GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACAAGTCCATTCGCACCAGGGC-3'; *PHOX2B*-gRNA, forward 5'-TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGATCTGGAATCCTGCGGCGG-3', reverse 5'-GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCGCCGCAGGATTCCAGATC-3'. The human codon optimized Cas9 expression vector (hCas9 plasmid, Addgene plasmid #41815) was purchased from Addgene. All insert sequences were verified by DNA sequencing (JHU Synthesis & Sequencing Facility).

hESC/hiPSC Culture and Neural Differentiation

We cultured undifferentiated H9 hESCs (passages 32-50), *OCT4::eGFP*, *SOX10::eGFP*, *ASCL1::eGFP*, and *PHOX2B::eGFP* genetic reporter H9 hESC lines (passages 42-60), control 01582, 02036, and 02623 iPSC lines (derived from GM01582, GM02036, and GM02623 fibroblasts (Coriell Institute), respectively) (passages 20-40), *PHOX2B::eGFP* genetic reporter 01582 hiPSC line (passages 40), on mitotically inactivated mouse embryonic fibroblasts (MEFs, Global Stem or Applied Stem Cell), in a medium containing DMEM/F12, 20% knockout serum replacement (KSR), 0.1 mM MEM-NEAA, 1 mM L-glutamine, 55 μM β-mercaptoethanol (Life Technologies) and 4 ng/mL FGF2 (R&D Systems) (hESC medium) as used routinely for hESC cultures (Lee et al., 2012). All cells were maintained at 37°C and 5% CO_2 in a humidified incubator. For neural differentiation, hPSCs were dissociated with Accutase. Differentiation media were described previously (Kriks et al., 2011): KSR and N2 medium for neural induction (day 0-10), and neurobasal medium supplemented with 2 mM L-glutamine, B-27 and N-2 supplements (Life

Technologies) for neuronal differentiation (after day 10). Neural induction using the LSB protocol was performed as previously described (Chambers et al., 2009; Chambers et al., 2011; Lee et al., 2010). Briefly, dissociated hESCs/hiPSCs were plated on Geltrex (Life Technologies) at a density of 20,000–25,000 cells/cm^2 in MEF-conditioned hESC medium containing 10 ng/mL FGF2 and 10 μM ROCK-inhibitor (Y-27632). Cells were allowed to reach 70%–80% confluence over 2 days. Differentiation was initiated by switching to medium that included KSR medium with 500 nM LDN193189 (abcam) and 10 μM SB431542 (Cayman Chemical). Beginning at day 4, the KSR medium was gradually replaced with increasing amounts of N2 medium (25% increments every other day). Other small-molecule compounds used in neural induction and differentiation were as follows: purmorphamine (PMP, 1 μM), ascorbic acid (0.2 mM), dibutyryl-cyclic AMP (dbcAMP, 0.2 mM), and PD173074 (PD, 0.2 μM) were purchased from Sigma-Aldrich; CHIR99021 (CHIR, 3 μM, Tocris), DAPT (10 μM, Cayman Chemical). Recombinant growth factors were as follows: Shh (C25II; 50–200 ng/mL), GDNF (10 ng/mL), and BMP4 (10 ng/mL) were purchased from R&D Systems; BDNF (10 ng/ml) and NGF (10 ng/mL) were purchased from PeproTech. All other chemicals were purchased from Sigma-Aldrich. After cell sorting or replating, the cells were cultured with the 'neuron medium', which contains neurobasal medium supplemented with 2 mM L-glutamine, B-27, N-2 supplements, 0.2 mM ascorbic acid, 0.2 mM dbcAMP, 10 ng/mL NGF, 10 ng/mL BDNF, and 10 ng/mL GDNF for further neural maturation.

Details of Culture Condition

Please see the Table S1 for more details about culture conditions in every assay we presented. The table contains the information of each Figure number, hPSC-derived neurons or others (source, final cell type, number of days since the pluripotent stage, number of days after lentiviral transduction), myocytes (source, final cell type, number of days after isolation or differentiation), and co-culture (number of days after co-culture).

FACS Analysis and FACS Sorting

For flow cytometry, cells were mechanically dissociated after exposure to Accutase, with 40 μg/mL DNase I (Roche Applied Science) for 20 min at 37°C. To eliminate dead cells in FACS analysis, 7-AAD (BD Pharmingen) was used according to the manufacturer's recommendations. Cells were analyzed by using BD FACSCalibur (Becton Dickinson) and FlowJo software (Tree Star Inc.), or sorted by using the MoFlo high-speed sorter (Dako Cytomation) in the Johns Hopkins School of Public Health Flow Cytometry Core Facility.

Immunofluorescence Staining

Cells were fixed in 4% paraformaldehyde and stained with the primary antibodies (listed below), after permeabilization with 0.1% Triton X-100/PBS solution. For visualization, we used appropriate Alexa Fluor® 488, 568, or 647 labeled secondary antibodies (Life Technologies or Jackson ImmunoResearch) and DAPI (Roche Applied Science) nuclear counter-staining. The stained samples were visualized using fluorescence microscopy (Eclipse TE2000-E, Nikon) or confocal microscopy (LSM710, Carl Zeiss). The color of Alexa Fluor® 647-stained images (originally far red) were converted into pseudo-green or pseudo-blue using NIS-Elements BR (Nikon), ZEN 2012 SP1 (Carl Zeiss), or Photoshop CS5 (Adobe). The primary antibodies used in this study are listed below with target, company (or gift from), catalog number (or reference), isotype, and dilution. ASCL1, BD Pharmingen, 556604, mouse IgG_1, 1/100; BRN3A, Chemicon, MAB1585, mouse IgG_1, 1/100; CHGA, ImmunoStar, 20086, rabbit IgG, 1/500; CHRNA7, Research & Diagnostic Antibodies, AS-5631S, rabbit IgG, 1/400; CHRNB4, Research & Diagnostic Antibodies, AS-5656S, rabbit IgG, 1/100; cTnT, Pierce, MA5-12960, mouse IgG_1, 1/500; cTnT (in Figure S4I), abcam, ab10214, mouse IgG_{2b}, 1/100; DBH, ImmunoStar, 22806, rabbit IgG, 1/500; GATA3, Santa Cruz Biotechnology, sc-268, mouse IgG_1, 1/100; GFP, abcam, ab13970, chicken IgY, 1/1000; HB9, DSHB, 81.5C10, mouse IgG_1, 1/50; INSM1, gift from Carmen Birchmeier (Welcker et al., 2013), rabbit IgG, 1/500; ISL1, DSHB, 40.2D6, mouse IgG_1, 1/50; MF20, DSHB, MF 20, mouse IgG_{2b}, 1/500; NANOG, Cell Signaling, #3580, rabbit IgG, 1/200; OCT3/4, Santa Cruz Biotechnology, sc-5279, mouse IgG_{2b}, 1/50; PHOX2B, gift from Jean-François Brunet (Pattyn et al., 1997), rabbit IgG, 1/800; PNMT, Enzo, PZ 1040, rabbit IgG, 1/200; PRPH, Santa Cruz Biotechnology, sc-7604, goat IgG, 1/100; RUNX1, abcam, ab23980, rabbit IgG, 1/200; SV2, DSHB, SV2, mouse IgG_1, 1/100; SYN1, Millipore, AB1543, Rabbit IgG, 1/1000; TH, Pel-Freez Biologicals, P40101-0, rabbit IgG, 1/200.

qRT-PCR and Microarray Analysis

Total RNA was extracted by using the TRIzol Reagent (Life Technologies), and reverse transcribed by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR mixtures were prepared with SYBR Green PCR Master Mix (Applied Biosystems) and reactions were done with the Mastercycler ep Realplex2 (Eppendorf). Each transcript levels were assessed by qRT-PCR normalized to *GAPDH* expression. Primers for qRT-

PCR were as follows: *ALK*, forward 5'-GGGCTGCTCCAGTTCAATCT-3', reverse 5'-CATCAGGCGGATCCTCAGTC-3'; *ASCL1*, forward 5'-AGCGCAGCCTTAGTAGGAGA-3', reverse 5'-AAGCAGCAGCAAAAGTCAGTG-3'; *CHAT*, forward 5'-GCCTTCTACAGGCTCCATCG-3', reverse 5'-GGAGTGGCCGATCTGATGTT-3'; *CHRNA3*, forward 5'-TCCTTCCCAGGGTGTGTTAG-3', reverse 5'-GGGACTCAGTTTCTGTCCCAG-3'; *CHRNA5*, forward 5'-ATCTGAACTGGACTAGTGAAAAATC-3', reverse 5'-TCCAGCACAGTCAAAGGATGA-3'; *CHRNB4*, forward 5'-CAGACCCATGCAGCTTCTGA-3', reverse 5'-CATCCTCTCACCCCACAACC-3'; *DBH*, forward 5'-AACGAGGATGTCTGCACCTG-3', reverse 5'-TTCAGTACGTCGCGGTTGAA-3'; *FAM134B*, forward 5'-AATCAGCAGCTGGTCTCACC-3', reverse 5'-TGCTGCACACCCTCTAACTG-3'; *FOXD3*, forward 5'-GACATGTTCGACAACGGCAG-3', reverse 5'-CTGTAAGCGCCGAAGCTCTG-3'; *GAPDH*, forward 5'-TCCAAAATCAAGTGGGGCGA-3', reverse 5'-AAATGAGCCCCAGCCTTCTC-3'; *GAPDH* (for single cell qRT-PCR), forward 5'-CCCACCACACTGAATCTCCC-3', reverse 5'-TACATGACAAGGTGCGGCTC-3'; *HAND2*, forward 5'-GGCAGAGATCAAGAAGACCGAC-3', reverse 5'-CGGCCTTTGGTTTTCTTGTCGTT-3'; *IKBKAP*, forward 5'-GCAGCAATCATGTGTCCCA-3', reverse 5'-ACCAGGGCTCGATGATGAA-3'; *INSM1*, forward 5'-CCTCGCCTACCAATCTCTGC-3', reverse 5'-TCCAGCAGTTCACAAGCCAT-3'; *ISL1*, forward 5'-TTGCCTCGGGAGCCCTAATC-3', reverse 5'-ATCATATTTCAGCCTCGCCGC-3'; *KCNJ14*, forward 5'-CCCTTCCCCAAGGTAGCAAG-3', reverse 5'-AGGCATGGCAGAACCTTACC-3'; *KIF1B*, forward 5'-GGAGTTGCCATTCGGGAAGA-3', reverse 5'-TGCTTGGCCAACCCTTGTAA-3'; *LMO1*, forward 5'-GAGACGGCCACGAGATTCC-3', reverse 5'-CTGGCAACGACACAGCTTTC-3'; *NANOG*, forward 5'-CAGAAGGCCTCAGCACCTAC-3', reverse 5'-ATTGGAAGGTTCCCAGTCGG-3'; *NGF*, forward 5'-CGTCCGGACCCAATAACAGT-3', reverse 5'-AGTGTGGTTCCGCCTGTATG-3'; *NTRK1*, forward 5'-TCTGGAGCTCCGTGATCTGA-3', reverse 5'-TGAGCCGAGGAGTGAAATGG-3'; *OCT4*, forward 5'-GGGGTTCTATTTGGGAAGGTAT-3', reverse 5'-TGTTGTCAGCTTCCTCCACC-3'; *PHOX2A*, forward 5'-CCGATGGACTACTCCTACCTCA-3', reverse 5'-GCAGGGGGCTGTATTGGAAG-3'; *PHOX2B*, forward 5'-CCAGTGCCAGCCCAATAGAC-3', reverse 5'-TGGACAATAGCAAAGCGGTTG-3'; *PNMT*, forward 5'-TACCTCCGCAACAACTACGC-3', reverse 5'-GGGCCTGAACCAATGTCGAT-3'; *PRDM14*, forward 5'-CTTTAATTCCACCGCCCCCT-3', reverse 5'-GGGTTTGGGACCATCCTCTG-3'; *RET*, forward 5'-CACCCGCCATCCAGACC-3', reverse 5'-GTAGAGGCCCAATGCCACTT-3'; *SCN2A*, forward 5'-ACAGCTTCCGCTTCTTTACCAG-3', reverse 5'-TGGGCCATTTTCATCATCCTCA-3'; *SDHA*, forward 5'-CATCTACGGAGCAGAGGCAC-3', reverse 5'-GCCAACGTCCACATAGGACA-3'; *SOX10*, forward 5'-CTCACTGCCCTGATGACCCA-3', reverse 5'-CAGCCCCTCATCTTTCAGTGT-3'; *TFAP2A*, forward 5'-ACCGACAACATTCCGATCCC-3', reverse 5'-CAGCAGGTCGGTGAACTCTT-3'; *TH*, forward 5'-TCATGTCCCCGCGGTTCATT-3', reverse 5'-TCCCCTCCTTCTCCTCAAAGG-3'; *VACHT*, forward 5'-GGCATAGCCCTAGTCGACAC-3', reverse 5'-CGTAGGCCACCGAATAGGAG-3'. Mouse *Chrna3* (*mChrna3*), forward 5'-TCCTGTCATCATCCAGTTTGAGG-3', reverse 5'-TCATTCCAGATTTGCTTCAGCC-3'; *mChrna5*, forward 5'-GAAGGGGCCAGTACGAAAACA-3', reverse 5'-AGCCGAATTTCATGGAGCAAT-3'; *mChrnb4*, forward 5'-TGGATGATCTCCTGAACAAAACC-3', reverse 5'-CAGGCGGTAGTCAGTCCATTC-3'; *mDbh*, forward 5'-AAGGTGGTTACTGTGCTCGC-3', reverse 5'-CACACATCTCCTCCAAGATTCC-3'; *mGapdh*, forward 5'-ATGTGTCCGTCGTGGATCTGA-3', reverse 5'-GCTGTTGAAGTCGCAGGAGACA-3'; *mGja5*, forward 5'-AGAGCCTGAAGAAGCCAACT-3', reverse 5'-GGCGTGGACACAAAGATGA-3'; *mHcn4*, forward 5'-TGGCTGATGGCTCCTATTTT-3', reverse 5'-GGCAATAAGTATCCGCTCTGA-3'; *mIrx4*, forward 5'-CAAGAGTGCCAAGAGCGAAG-3', reverse 5'-ACATTCTGAGGTCTCTGCATCT-3'; *mMyh6*, forward 5'-TGGTGGACAAGCTACAGTTGAAGG-3', reverse 5'-CGGAACTTGGACAGGTTGGTG-3'; *mMyl2*, forward 5'-GACGGCTTCATCGACAAGAATG-3', reverse 5'-TCCCCAAACATCGTGAGGAAC-3'; *mMyl7*, forward 5'-CATGACCCAGGCAGACAAGTTC-3', reverse 5'-CCGTGGGTGATGATGTAGCAG-3'; *mScn5a*, forward 5'-CCGCCCTATAGTGCTGGAC-3', reverse 5'-TCCTCAAAGCCATCTGCAC-3'; *mTh*, forward 5'-AGTTCTCCCAGGACATTGGACTT-3', reverse 5'-ACACAGCCCAAACTCCACAGT-3'. For microarray, total RNA from each population was processed by the JHMI Deep Sequencing & Microarray Core Facility and hybridized on the PrimeView Human Gene Expression Arrays (Affymetrix). The microarray data were analyzed and visualized using Partek Genomics Suite (Partek Inc.). Transcripts up-regulated at least 2-fold ($P < 0.001$) were used for gene ontology (GO) analysis with DAVID (http://david.abcc.ncifcrf.gov). All microarray data used in this study have been deposited to NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) under accession number GEO: GSE80689.

Single Cell qRT-PCR

This protocol followed Pan et al.'s procedure for cDNA synthesis and amplification (Pan et al., 2013). Briefly, the single cell was isolated by fluorescence-activated cell sorting (FACS) for cells expressing PHOX2B::eGFP and lysed directly using cell lysis buffer (Ambion). The mRNA was selected from total RNA in Reverse Transcription (RT) using SMA-T15 (5'-GACATGTATCCGGATGTTTTTTTTTTTTTTT-3'), with anchoring nucleotide at the 5′ end SMA-A (5'-ACATGTATCCGGATGTGGG-3'). The thermal program was set as 25 °C × 10 min, 42 °C × 90 min, and 85 °C × 5 min. The excess oligonucleotides SMA-A and SMA-T15 were digested with ExoSAP-IT reagent (USB). The product from the RT reaction was PCR amplified using universal sequence as SMA-p2 primer (5'-GACATGTATCCGGATGT-3'). The amplification consisted of 20 cycles of denaturation (94°C for 20 s), annealing (57°C for 20 s), and extension (68°C for 7 min). qRT-PCR mixtures were prepared with SYBR Green PCR Master Mix (Kapa Biosystems), and done in technical duplicates with the Eppendorf realplex2.

Whole-Cell Electrophysiology Recordings

hESC-derived sympathetic neurons were cultured on coverslips coated with PO/Lam/FN (poly-L-ornithine hydrobromide/laminin/fibronectin) (PO and Lam from Sigma-Aldrich, FN from BD Biosciences) for 14 days with neuron medium. Then a coverslip was transferred into a perfusion chamber with extracellular solution (ECS) containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH adjusted to 7.4 with NaOH, osmolarity adjusted to 300 mOsm with sucrose. Electrodes were pulled (Model pp-830, Narishige) from borosilicate glass (WPI Inc.) and had resistance of 2-4 MΩ. The pipette was filled with internal solution containing 135 mM KCl, 1.1 mM $CaCl_2$, 10 mM HEPES, 2 mM EGTA, 3 mM Mg-ATP, 0.5 mM Na_4GTP, pH adjusted to 7.3 with KOH, osmolarity adjusted to 290 mOsm with sucrose. With voltage-clamp whole-cell configuration (holding potential -60 mV), currents were measured with an Axon 700B amplifier and the pCLAMP 9.2 software package (Molecular Devices) in order to characterize I-V relation. With current-clamp whole cell configuration, a train of current from 100 pA to 800 pA (with an increment of 100 pA and an interval of 4 s) was injected for 200 ms to elicit action potentials. High potassium solution (50 mM KCl) was also perfused to elicit depolarization. To detect afterhyperpolarization (AHP), a 300 pA current was injected during 10 ms to elicit action potentials.

Electrophysiological Verification of Optogenetic Stimulation

ESC-derived sympathetic neurons were targeted for whole-cell patch clamp recording with borosilicate electrodes (3–6 MΩ) at room temperature in HEPES-based ACSF containing 143 mM NaCl, 10 mM HEPES, 5 mM KCl, 10mM D-glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4. The pipette was filled with internal solution containing 135 mM KCl, 1.1 mM $CaCl_2$, 10 mM HEPES, 2 mM EGTA, 3 mM Mg-ATP, 0.5 mM Na_4GTP, pH adjusted to 7.3 with KOH, osmolarity adjusted to 290 mOsm with sucrose. The junction potential was left uncorrected. Signals were measured with a MultiClamp 700B amplifier and digitized using a Digidata 1440A analog-to-digital board (Molecular Devices). Data was acquired with pClamp 10 software and digitized at 20 kHz. Neurons were optogenetically stimulated with a blue LED (470nm, Thorlabs). Light intensity was calibrated at the back aperture of the 40 × 0.8 NA Zeiss objective.

Calcium Imaging

hESC-derived sympathetic neurons with or without neonatal mouse ventricular myocytes (NMVM) were loaded with Fura-2-acetoxymethyl ester (Molecular Probes) for 30 min in the dark at room temperature. After rinsing with calcium imaging buffer, cells were imaged at 340 and 380 nm excitation to detect intracellular free calcium concentration upon perfusion of 0.1 or 1 μM (-)-nicotine hemisulfate (Sigma-Aldrich) dissolved in the imaging buffer. The calcium imaging buffer consists of 130 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 0.6 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 1.2 mM $NaHCO_3$, with pH adjusted to 7.4 using NaOH and osmolarity adjusted to 290 mOsm using sucrose. Data were described as the ratio of Fura-2 light emission on excitation at 340 and 380 nm (340/380) or the normalized ratio ($\Delta F/F_0$; $\Delta F = (F - F_0)$, F = the 340/380 at a given time point, F_0 = the mean basal, unstimulated 340/380 of each cell). A typical non-response area was selected for fluorescence bleed normalization and background subtraction. Neurons were defined as the cells with round-shaped cell bodies extending at least two neurites that were longer than the diameter of the cell body. Fura-2 was predominantly stained at the neuronal cell bodies rather than cardiomyocytes when co-cultured cardiomyocytes with neurons. The threshold for a positive calcium response to the addition of the nicotine was determined as $\Delta F/F_0 > 0.10$, which represents the mean plus 10-fold standard deviation from $\Delta F/F_0$ recordings in the period of no stimulation. Calcium-imaging assays were performed with an experimenter blind to treatment. The data were analyzed using Sigma Plot 11 (Systat Software Inc.) and Prism (GraphPad).

Catecholamine ELISA

After washing with HBSS (Life Technologies), cultured hPSC-derived sympathetic neurons incubated with a HBSS for 15 min. The media were collected as a control. Then the cells incubated with 50 mM KCl in HBSS for 15 min. For detecting photostimulation-induced norepinephrine release, after washing with HBSS, cultured hESC-derived hChR2-expressing sympathetic neurons incubated with a HBSS for 15 min in dark. The media were collected as a control. Then the cells in a new HBSS incubated with continuous wide-field blue light (26 mW/mm^2 at 488 nm), or no illumination as a control, for 15 min. Following to media collection, the media were centrifuged at 300 g for 5 min to eliminate cells or debris. To prevent catecholamine degradation, 1 mM EDTA and 4 mM sodium metabisulfite were immediately added to the samples and stored at -80°C until analyzed. Total catecholamine levels in the samples were quantified with a catecholamine ELISA kit (Abnova) according to manufacturer's instruction. To determine the levels of catecholamine release in each type of cultured neuron, calculated levels of catecholamines in the HBSS control were subtracted from 50 mM KCl (or blue light illuminated) samples.

Differentiation of Cardiomyocytes from hiPSC or mESC

For differentiation human iPSCs (hiPSCs) into cardiomyocytes, 02623 hiPSCs were cultured in Essential 8 Medium and dissociated by TrypLE (Gibco), and then 1.5×10^6 cells were plated in Matrigel (Corning) coated 6 well plate. After 24 hours, changed the medium with a RB medium (RPMI 1640 supplemented with B-27 without insulin) containing 6 μM CHIR99021 and then changed the medium with RB medium containing 10 μM XAV939, and then medium was changed every 2 days with. At day 14, differentiated cardiomyocytes were dissociated by TrypLE to make single cardiomyocyte. Then, $2–3 \times 10^5$ hiPSC-derived cardiomyocytes were added onto hESC-derived sympathetic neurons (14 days after PHOX2B::eGFP+ sorting) with 1:1 mixed medium (RPMI 1640 supplemented with B-27 : neuron medium). For differentiation mouse ESCs (mESCs) into cardiomyocytes, *Isl1-RFP/Myh6-GFP* mESC line (Shenje et al., 2014) was differentiated in SFD medium, which contains IMDM and Ham's F12 in a 3:1 ratio supplemented with 0.5% N-2 supplements, 1% B-27 supplements, 0.05% BSA, 1.5 mM L-glutamine, 0.75% penicillin/streptomycin, 50 μg/mL ascorbic acid, and 0.039% monothioglycerol, as described previously (Gadue et al., 2006; Kattman et al., 2011). In brief, mESCs were dissociated with TrypLE and cultured in SFD medium (100,000 cells/mL). After 48 hours, human Activin A (5 ng/mL), human BMP4 (0.7 ng/mL) and human VEGF (5 ng/mL) were added (R&D Systems). SFD medium was changed every other day. Isl-RFP expression was detected around day 4-5 and Myh6-GFP expression was detected around day 6-7 with beating. Isl1+/Myh6+ mESC-derived cardiomyocytes were then collected by using SH800 sorter (Sony Biotechnology). The resulting cardiomyocytes were cultured with mouse cardiomyocytes medium, which contains DMEM and Medium 199 in a 4:1 ratio supplemented with 10% FBS and 1% penicillin/streptomycin. Then, $2–3 \times 10^5$ FACS-purified mESC-derived cardiomyocytes were added onto hESC-derived sympathetic neurons (5 days after PHOX2B::eGFP+ sorting) with 1:1 mixed medium (mouse cardiomyocytes medium : neuron medium).

Preparation of Skeletal Myotubes from C2C12 Cells

C2C12 mouse adherent myoblasts were grown in DMEM supplemented with 20% FBS and 1% penicillin/streptomycin. After reaching 80% confluence, the medium containing 20% FBS was substituted with 2% horse serum (C2C12 differentiation medium) to differentiate C2C12 myoblasts toward skeletal myotubes. Two days after differentiation, 1×10^6 trypsinized cells were added onto hESC-derived sympathetic neurons (14 days after PHOX2B::eGFP+ sorting) with 1:1 mixed medium (C2C12 differentiation medium : neuron medium).

Transmission Electron Microscopy (TEM)

For transmission electron microscopy, the JHMI Microscope Facility processed the samples. Briefly, the samples were fixed in 2.5% glutaraldehyde, 3 mM $MgCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.2 for one hour at room temperature. After buffer rinse, samples were post-fixed in 1% osmium tetroxide in buffer (1 h) on ice in the dark. Following a distilled water rinse, coverslips were stained with 2% aqueous uranyl acetate (0.22 μm filtered, 1 h, dark), dehydrated in a graded series of ethanol and embedded in Eponate 12 (Ted Pella) resin. Samples were polymerized at 60°C overnight and coverslips removed under liquid nitrogen. Thin sections, 60 to 90 nm, were cut with a diamond knife on the Ultracut E ultramicrotome (Reichert-Jung) and picked up with 2 × 1 mm copper slot grids. Grids were stained with 2% uranyl acetate in 50% methanol and observed with a Hitachi 7600 TEM at 80 kV.

High Speed Video Image Acquisitions with or without Multi-Electrode Array (MEA) Recording

A high speed digital CMOS camera (KP-FM400WCL, Hitachi) was mounted on an inverted microscope (Eclipse Ti, Nikon). Movie images of beating NMVM were recorded as sequential phase-contrast images with a 10 × or 20 × objective at a frame rate of 150 fps, a resolution of 2048 × 2048 pixels and a depth of 8 bits. MED probes (MED-

P515A, Alpha MED Sciences) equipped with platinum black-coated 64 planar microelectrodes that were arranged in an 8 × 8 grid embedded in the center of a transparent glass plate were used for extracellular recordings of the field potential (FP) from the NMVM. Image acquisitions and multi-electrode array (MEA) recordings were synchronized using external triggering options of the MEA system.

Optical Mapping of Action Potentials

Action potentials of co-cultured samples were recorded by staining with 10 μM di-4-ANEPPS (voltage sensitive dye, ThermoFisher Scientific) and imaging with MiCAM Ultima-L camera (SciMedia) at 500 frames per second. Beating of cardiomyocytes were suppressed by 10 μM blebbistatin (Sigma-Aldrich) to reduce artifact in optical action potentials due to motion. Electrical stimulation at 500 ms cycle length was applied throughout the experiment through a pair of platinum electrode placed at the edge of the sample. Action potentials of the sample were calculated by averaging signals throughout the field of view (2 mm × 2 mm), and the action potential duration was calculated by the time from upstroke (defined as time of maximum upstroke velocity) to 80% repolarization (APD80).

Statistical Analysis

Values are from at least three independent experiments with multiple replicates each, and reported as mean ± S.E.M. or mean + S.E.M. Differences between two samples were analyzed for significance using the unpaired Student's *t*-test in Sigma Plot 11. Differences between more than two groups were analyzed for significance using the ANOVA test in the Partek Genomics Suite.

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